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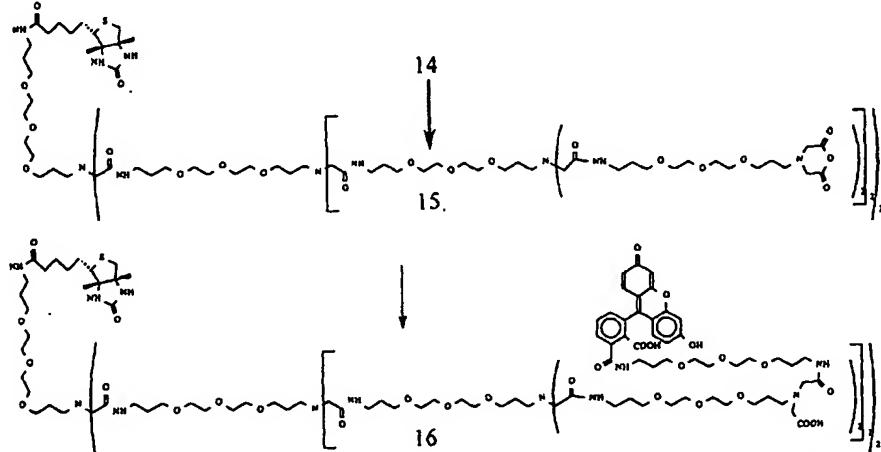
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(54) Title: DETECTION USING DENDRIMERS BEARING LABELS AND PROBES



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(57) Abstract: Novel dendrimers as well as novel dendrimer complexes are disclosed. Such dendrimers and/or dendrimer complexes may be used for the detection of various components of a sample and as detection systems and signal enhancement/amplification systems. The dendrimers and dendrimer complexes may also be used for labelling various entities/compounds. Furthermore, labelling kits and detection kits comprising one or more labelled dendrimers or one or more dendrimer complexes are also one of the possible uses.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DETECTION USING DENDRIMERS BEARING LABELS AND PROBES

FIELD OF THE INVENTION

5 The present invention relates to the field of polymer chemistry and, in particular to novel dendrimers as well as novel dendrimer complexes. In a further aspect, the invention concerns fields of use for such dendrimers and/or dendrimer complexes in the detection of various
10 components of a sample, as detection systems, signal enhancement/amplification systems, and labelling systems. Furthermore, kits comprising the dendrimers/dendrimer complexes are also part of the present invention.

15 BACKGROUND OF THE INVENTION

Probes that allow for the detection of specific biological markers such as proteins and nucleic acids are of wide-spread use and are a valuable tool for both *in vitro* and *in vivo* diagnostic purposes as well as in life-science research. However, detection of these markers can be difficult as not all are present in a sufficiently high number. This makes the detection of low copy number targets with a single labelled probe impossible or
25 impractical even though the probe may hybridise to the target with a high degree of specificity and fidelity. This substantial limitation has facilitated the development of various detection systems, the purpose of which is to enhance the signal that is detectable in an
30 assay. Examples of such systems include both target amplification (e.g. PCR, NASBA, SDA and LCR) as well as signal amplification techniques (e.g. Tyramide Signal Amplification).

35 Despite of the many different detection systems available, there still exists an opportunity for

improvement. Dendrimers presents one such opportunity to improve detectable signal in assays employing targets of low copy number because single probes can be modified with multiple detectable labels as a means to increase 5 signal output from the probe.

In general, dendrimers are well-defined macromolecules that diverge from a focal point through multiple branching points to terminate in multiple peripheral 10 functional groups. These peripheral functional groups can be used to link multiple labels (e.g. biotin, fluorophores, or combinations thereof) to other molecules such as DNA oligomers. Alternatively, multiple 15 macromolecules such as peptides or nucleic acids, or a combination of the two, can be linked to the dendrimers. The ability to link the same or different molecules of choice at the periphery of the dendrimer provides for signal amplification potential.

20 For example, dendrimers have been used to improve an immune response (e.g. in vaccination strategies) by facilitating the presentation of antigens or antigenic peptides to the immune system. Specifically, Multiple 25 Antigenic Peptides (MAPs) and Antigen Presenting Cells (APCs) are related systems where multiple peptides are synthesised or grafted upon a small polylysine core in order to enhance the antigenic properties relative to the individual peptides.

30 The macromolecules applied to dendrimer formation typically included linear macromolecules (e.g. dextran), branched macromolecules (e.g. hydrolysed starch), cyclic 35 macromolecules (e.g. cyclodextrans) and spherical macromolecules (e.g. nanoparticles). They have in particular been used to display multiple probes such as

antibodies, or signal generating proteins such as HRP, or combinations thereof.

Notwithstanding their prevalent use, the known dendrimers 5 have several drawbacks and limitations. For example, most of the known dendrimers are very compact structures giving raise to significant steric hindrance or crowding in the outer layers (periphery) of the dendrimer. This is especially pronounced in the case of MAPs, since there 10 are only 3 or 6 bonds between branching points. The well known StarBurst™ dendrimers comprise only 7 bonds between branching points and therefore soon reach a situation where there is little or no free space available for conjugation at the periphery of the 15 dendrimer; even for such small molecules (labels) such as biotin (ref. 1). With such little available space at the periphery, larger molecules such as DNA oligomers are particularly difficult to conjugate to the dendrimer; and if successfully conjugated, the kinetics of hybridisation 20 to the oligomer are limited by the crowded periphery of the macromolecular complex.

For example, dendrimers of DNA oligomers have been prepared. These are, however, very large and bulky 25 molecules. While the spacing between branching points can be several hundred bonds, the double helices that hold the structure together are very bulky relative to their longitudinal extension. Large bulky molecules can also be difficult to work with in certain applications since they 30 can exhibit reduced cellular uptake and mobility.

Yet another limitation of conventional dendrimers is that they typically must be prepared by solid phase chemistry if the impurities are to be successfully removed. In 35 particular, MAPs are almost always prepared by solid phase techniques, thus, limiting this technique to

smaller linear peptides as larger biomolecules in general are incompatible with the harsh cleavage/deprotection conditions.

5 Whilst still another drawback of conventional dendrimers is that the vast majority of dendrimers are homo-functional. Because they are homofunctional, it can be difficult or impossible to attach different ligand to the dendrimer in a well-defined manner.

10 Because of the crowding, specific types of signal amplification can be difficult to achieve in conventional dendrimers. For example, when multiple (identical or different) fluorophores are linked to dendrimers having a 15 crowded periphery, the fluorophores are likely to quench each other by collision induced or by quantum transfer of energy or electrons where the efficiency of quenching is proportional to the sixth power of distance between the fluorophores. This quenching limits signal output since 20 the large number of fluorophores produce a signal that is significantly less than the sum of the signal possible from each of the individual fluorophores and thereby thwarts the concept of linking multiple fluorophores to a dendrimer to thereby achieve substantial improvement in 25 signal output from a single species (e.g. a DNA probe).

Background straining is another well-known problem in detection. In the field of multi-layer visualisation systems, the potential background problem increases with 30 the number of layers and their chemical diversity. Blocking and stringent washing conditions must be optimised for each layer, however, still be compatible with prior and subsequent layers, thus, limiting the construction of multi-layer systems.

35

SUMMARY OF THE INVENTION

By the present invention, novel dendrimers and dendrimer complexes are provided which do not possess the above-mentioned drawbacks and limitations. The dendrimers and 5 dendrimer complexes of the invention provide spacing between the terminal peripheral functional groups to which various compounds are attached. Thus, the dendrimers and dendrimer complexes of the present invention have a less compact or crowded structure, 10 particularly at the periphery, as compared with the known dendrimers. Indeed, the dendrimers of the present invention are of low molecular weight as compared to their spatial size and are therefore less bulky as compared with conventional dendrimers such as MAPs and 15 APCs. Furthermore, the dendrimers of the present invention can be prepared by liquid phase chemistry.

Finally, and of significant importance, the dendrimers of the present invention are well-defined in molecular 20 structure and are heteromultifunctional, i.e. the dendrimer/dendrimer complex enable at least two different substituents/entities, and further enable presentation of at least one of these in multiple copies. Consequently, many combinations of different moieties can be linked to 25 the periphery of the dendrimers of the present invention is a highly defined manner and with a highly defined stoichiometry. Thus, the dendrimers of the present invention can be tailor made for specific applications and will not suffer from lot to lot variations commonly 30 observed with other polymeric conjugates. Therefore, the dendrimers and dendrimer complexes of the present invention are useful in a wide range of applications, e.g. as detection systems and signal enhancement systems.

35 In a first aspect, the present invention relates to novel dendrimers having the general formula (I)

(Entity1)_{x1}-A-(Entity2)_{x2} (I)

wherein A is a dendritic core having at least one N-
5 atomic branching point, said branching point not being
part of a naturally occurring amino acid, and wherein A
comprises at least one ether group,
each Entity1 and Entity2 are independently a probe, a
probe substituted by one or more labelling compounds, a
10 labelling compound, or a probe having reactive groups,
x1 and x2 are independently 0 or an integer of from 1 to
1200,
and protected forms thereof.

15 In another aspect, the present invention relates to novel
dendrimer complexes and protected forms thereof
comprising at least one dendrimer as defined herein,
which dendrimer or dendrimers are connected to other
20 compounds of interest, e.g. a naturally or non-naturally
amino acid terminally or internally, a peptide nucleic
acid terminally or internally, an LNA terminally or
internally, a peptide terminally or internally, a protein
terminally or internally, an antibody, an antigen, an
immune complex, a RNA sequence or an analogue thereof
25 terminally or internally, a DNA sequence or an analogue
terminally or internally, a macromolecule terminally or
internally, or a solid or semi-solid support.

30 The dendrimers and dendrimer complexes as well as the
protected forms thereof of the present invention have a
broad range of possible uses including the use in the
detection of the presence of various components of a
sample, like the detection of nucleic acid sequences,
35 antibodies, antigens, immune complexes, proteins, or
peptides.

Furthermore, the dendrimers and dendrimer complexes of the present invention may be used as or in detection systems and signal amplification systems. Such systems may suitably be used in the detection of various 5 components of a sample, including the detection of nucleic acid sequences, antibodies, antigens, immune complexes, proteins, or peptides.

10 Also, the dendrimers and dendrimer complexes of the present invention enable the application in *in vitro* and *in vivo* diagnostic methods. Accordingly, the present invention i.a. relates to detection kits and amplification kits comprising such dendrimers and dendrimer complexes and/or protected forms thereof.

15 Another possible use of the dendrimers and dendrimer complexes as well as the protected forms thereof of the present invention is in the labelling of various compounds. Thus, the present invention further relates to 20 the use of the dendrimers and dendrimer complexes as well as the protected forms thereof in labelling reactions as well as to labelling kits comprising such dendrimers and/or dendrimer complexes, and/or protected forms thereof.

25 The present invention is described in detail in the following.

BRIEF DESCRIPTION OF THE FIGURES

30 Figure 1 shows examples of various suitable protecting groups.

Figure 2 shows examples of activated groups.

35 Figure 3 shows examples of free groups.

Figures 4-17 shows the various dendrimers of the Examples.

5 DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention relates to dendrimers having the general formula (I)



wherein A is a dendritic core having at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein A
15 comprises at least one ether group,
each Entity1 and Entity2 are independently a probe, a probe substituted by one or more labelling compounds, a labelling compound, or a probe having reactive groups,
x1 and x2 are independently 0 or an integer of from 1 to
20 1200,
and protected forms thereof.

From WO 98/32469 (ref. 2), compounds comprising a linear, branched or dendrimeric polymer backbone comprising a plurality of amine-containing acids are known. In particular, the polymeric backbone is composed of native or non-native amino acid residues. Such compounds have linked thereto at least one reporter moiety. The polymers (dendrimers) may be asymmetrical or symmetrical. The
25 dendrimers are useful as therapeutic and diagnostic agents. The dendrimers of the present invention differ from the dendrimers disclosed in WO 98/32469 in that the dendritic core has at least one N-atomic branching point that is not part of a naturally occurring amino acid, and
30 further in that the dendritic core comprises at least one ether group.

From EP 271 180 (ref. 3), and Bioconjugate Chem. 9, 813-825 (1998) (ref. 4), so-called StarBurst™ dendrimers are known. Such dendrimers are dense structures exhibiting a molecular architecture characterised by regular dendritic 5 branching with radial symmetry. The StartBurst™ dendrimers are suitable for carrying various materials. The dendrimers of the present invention differ from the dendrimers disclosed in EP 271 180 (ref. 3) and Bioconjugate Chem. (ref. 4) in that the dendritic core 10 comprises at least one ether group, and further in that the dendrimers of the present invention are heteromultifunctional, whereas all peripheral groups of the conventional StarBurst™ dendrimers are identical.

15 From WO 97/07398 (ref. 5), dendrimer/polypeptide complexes are known. The complex comprises a dendrimer with a plurality of termini, having coupled thereto a first and a second polypeptide, whereby the formed complex exhibits first and second defined biological 20 activities. Also comprised is dendrimer/polypeptide complexes, wherein a first and a second are coupled to each other. The dendrimers to be used in accordance with WO 97/07398 (ref. 5) are not defined specifically as the invention as defined in this document does not seem to 25 lie in the dendrimers as such. However, from the description, it appears that the StarBurst™ dendrimers are suitable. As mentioned above, the dendrimers of the present invention differ from the StarBurst™ dendrimers in that the dendritic core comprises at least one ether 30 group, and further in that the dendrimers of the present invention are heteromultifunctional, whereas all peripheral groups of the conventional StarBurst™ dendrimers are identical.

35 The StarBurst™ dendrimers are dense structures exhibiting "starburst dense packing", where the surface

of the dendrimer contains sufficient terminal moieties such that the dendrimer surface becomes congested and encloses void spaces within the interior of the dendrimers, and this congestion can provide a molecular 5 level barrier which can be used to control diffusion of material into or out of the interior of the dendrimer, cf. page 6, lines 11-19 of EP 271 180 (ref. 3). This so-called "starburst topology" is achieved by assembling organic repeating units in concentric, dendritic tiers 10 around an initiator core, cf. page 2, lines 20-31 of EP 271 180 (ref. 3). The topology is achieved by forming successive layers of relatively short amino-containing chain units, in particular the PEI and PAMAM, cf. 31 and the examples of EP 271 180 (ref. 3). As mentioned, this 15 leads to very dense structures, and the materials to be carried may have to carry connectors and/or spacers to facilitate the use of the starburst conjugates, cf. page 13, line 29 to page 14, line 1 of EP 271 180 (ref. 3).

20 In WO 98/32469 (ref. 2), it is mentioned that the invention lies in the recognition that co-polymers of amino acids carrying or attached to one or more reporter groups are particularly suitable for diagnostic and therapeutic use, cf. page 4, third paragraph. As 25 mentioned on page 6, second paragraph of WO 98/32469 (ref. 2), the dendrimers comprise a plurality of native or non-native amino acids. It can be derived from page 7, third paragraph of WO 98/32469 (ref. 2) that the use of amino acids results in that the dendrimer becomes 30 biodegradable. The dendrimer may also comprise other moieties, cf. page 6, third paragraph of WO 98/32469 (ref. 2). Such other moieties are relatively short amino-containing chains. The use of successive layer of amino acids and/or other moieties results in dendrimers with a 35 very dense structure.

As mentioned in the introductory part of this description, such dense dendrimer structures have several drawbacks, i.a. significant steric hindrance and crowding in the outer layer of the dendrimer, and quenching of 5 fluorophores attached to the outer layers of the dendrimer. In contrast hereto, the dendrimers and dendrimer complexes of the present invention do not possess these drawbacks. Thus, the advantages of the dendrimers and dendrimer complexes of the present 10 invention are numerous. They have a sufficiently loose structure to allow conjugation of even quite large entities. Furthermore, the closest neighbouring anchoring groups are sufficiently far apart, whereby reduced reactivity, aggregation of the attached entities, 15 fluorescence quenching and other undesirable effects of steric crowding are avoided or minimised. Furthermore, another pronounced advantage of the dendrimers and dendrimer complexes of the present invention is that they generally have good solubility due to a content of 20 hydrophilic groups. Also, the dendrimers are easily derivatised with a desired entity, e.g. a probe or a labelling compound, whereby the full potential of the multiple sites can be fully exploited, and well-defined conjugates prepared. Furthermore, it is possible to 25 activate the dendrimer in advance, so that chemical modifications of the attached entities are avoided, and naturally occurring functionalities such as amines, carboxylic acids, thiols, alcohols etc. can be brought to react spontaneously with the dendrimer. In particular, 30 the dendrimer may be heterofunctional. In this way, one type of entity, e.g. a probe or a labelling compound, can be attached to one or some groups on the dendrimer, while other entities, e.g. other probes or labelling compounds, can be attached to other groups using orthogonal 35 chemistries, the exact stoichiometry being controlled by different chemistries, not by having different entities

competing for the same sites. For example, dendrimers having 17 bonds between branching points can be constructed wherein there at the periphery are 40 bonds between nearest neighbouring anchoring groups. The 5 dendrimers can e.g. be derivatised as a mono Boc-amino polyanhydride, or polyBoc-amino mono anhydride. An advantage of the present dendrimers is their easy derivatisation with peripheral imodiacetic acid anhydride moieties. This moiety reacts very fast and selectively 10 with primary aliphatic amines allowing conjugation in water under aqueous conditions with unprotected nucleic acids or analogues thereof such as peptide nucleic acids. Conversely, the Boc group can be removed and the resulting free amino group can be reacted directly with 15 activated carboxylic acids, can be reacted to yield malimides or 2-haloacetyls that are thiol reactive, or can be reacted to give an anhydride that again is amino-reactive. Beneficial is the complete orthogonality obtained between multiple benzyl ester groups, and a Boc- 20 amino group. Thus, herterofunctional dendrimers can readily be prepared.

In the dendrimers of the present invention, the dendritic core A extends from a single focal point through multiple 25 generations of successive layers, each layer having one or more branching points, having regard to the definitions above.

In particular, the dendritic core A comprises one or more 30 of C₁₋₁₀₀ alkyl groups, C₂₋₁₀₀ alkenyl groups, C₂₋₁₀₀ alkynyl groups, said alkyl, alkenyl, and alkynyl groups optionally containing one or more functional groups and/or one or more heteroatoms, naturally or non-naturally amino acids, peptide nucleic acid moieties, 35 LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA

sequences or analogues thereof, macromolecules, and solid or semi-solid supports, however, having regard to the provisos above, i.e. that the dendritic core has at least one N-atomic branching point, said branching point not 5 being part of a naturally occurring amino acid, and wherein A comprises at least one ether group. It is to be understood that each layer may comprise one or more of the residues mentioned.

10 The alkyl, alkenyl and alkynyl groups may in particular be such with C₅₋₈₀, C₅₋₇₀, C₅₋₆₀, C₁₀₋₅₀, C₁₀₋₂₅, C₁₅₋₂₅, and C₁₀₋₂₀ carbon atoms which further optionally contains one or more functional groups and/or one or more heteroatoms. The alkyl, alkenyl and groups may be straight chain or 15 branched groups.

The term "functional group" is intended to comprise groups such as e.g. ester groups, ether groups, thiol groups, carbonyl groups, hydroxyl groups, amide groups, 20 carboxylic groups, and imide groups as well as combinations thereof.

The term "heteroatoms" is intended to include i.a. O, N, S and P.

25 Within the present context, the term "probe" is intended to mean a compound of chemical or biological origin that specifically recognises and binds to markers and/or complexes thereof. Several probes can be envisaged. Ways 30 of selecting probes targeting a desired marker or nucleic acid sequence are known to the person skilled in the art. Suitable probe sequences include i.a. such originating from bacteria, viruses, fungi, allergens, chromosome and markers. It is within the scope of the present that 35 several probes may be connected to each other. Thus, the expression "a probe" is intended to include a combination

of probes which may be connected to each other terminally or internally.

The terms "RNA" and "DNA" are of course well known expressions and it is accordingly believed that no specific references need to be mentioned. The term "DNA" further comprise both the sense and the anti-sense strands. Analogues of RNA and DNA is intended to comprise any chemical modification of such, e.g. modified linkages (like phosphothioates), modified riboses (like LNA) or modified bases (e.g. 5-substituted uracils). "Peptide nucleic acids" are synthetic molecules in some aspects resembling the functions of DNA. Peptide nucleic acids were firstly disclosed in WO 92/20702 (ref. 6) and WO 92/20703 (ref. 7). The term "peptide nucleic acid" is to be interpreted broadly as it conventionally used to characterise compounds having a polymeric backbone, cf. the definition of WO 92/20702 (ref. 6) and WO 92/20703 (ref. 7). "LNAs" are newly invented compounds also in some aspects resembling the functions of DNA. "Peptides", "proteins", "antibodies", "antigens" and "immune complexes" are well known terms, and is within the present context intended to include any such suitable for the purposes of the present invention. The antibodies and immune complexes may be used as monomers, dimers, trimers, tetramers, or various multimers/aggregates. Also included are antibodies/immune complexes of from the classes IgA, IgD, IgE, IgG and IgM as well as subclasses thereof. The term "antibody" also comprises epitopes, and various fragments of such antibodies, e.g. Fab fragments and single-chain fragments. Also comprised are monoclonal, polyclonal, and recombinant antibodies. The term "macromolecules" is intended to include dextrans, polyvinylpyrrolidone, branched or linear polyethers, and branched or linear polylysines. Within the present context, the term "solid or semi-solid support" is

intended to include particles and beads e.g. of polystyrenes, polypropylenes, polyethylenes, dextrans, nylon, amyloses, celluloses, polyacrylamides and agarose, membranes e.g. of cellulose, cellulose acetate, glycerols 5 and polyvinylidene fluoride.

Both the dendrimers as well as the protected forms of such dendrimers are aspects of the present invention. A protected form is such having protecting groups attached 10 thereof. The dendritic core itself may be protected by protecting groups (e.g. in the case where x_1 or x_2 , or x_1 and x_2 are 0). Such protecting groups may be located at the outermost layer of the dendritic core. Also, Entity1 and Entity2 may independently be

15 a protecting group,

a probe protected by a protecting group (a protected probe),

a labelling compound protected by a protecting group (a protected labelling compound),

20 a protected probe substituted by one or more labelling compounds,

a protected probe substituted by one or more protected labelling compounds,

25 a probe having reactive group protected by a protecting group (a protected reactive group).

Examples of suitable protecting groups are Fmoc, Boc, Mtt, Mmt, Dde, All, Aloc, ODmab, OtBu, Ome, Obz, Z, MOM and benzyloxycarbonyl.

30 The dendritic core may terminate in a free group, a reactive group or an activated group (e.g. when x_1 or x_2 or x_1 and x_2 are 0). A few examples are amines and acids.

35 In a preferred embodiment, the dendritic core A may in each consecutive layer comprise one or more of

$-\text{NH}((\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{N}((\text{CH}_2)_q\text{C}(\text{O})-)_2,$

$((-\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{N}((\text{CH}_2)_q\text{O}-)_2,$

$((-\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{C}((\text{CH}_2)_q\text{O}-)_3,$ and

$-\text{NH}((\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{C}((\text{CH}_2)_q\text{C}(\text{O})-)_3,$

5 wherein each q independently is 0 an integer from 1 to 8, preferably from 1 to 3. Also comprised is successive layers containing one or more of these specified groups as well as one or more of naturally or non-naturally amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA sequences or analogues thereof, macromolecules, and solid or semi-solid supports, and further layers containing one or more of these specified groups and other layers 10 containing one or more of naturally or non-naturally amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA sequences or analogues thereof, macromolecules, and solid 15 or semi-solid supports, and further layers containing one or more of naturally or non-naturally amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA sequences or analogues thereof, macromolecules, and solid 20 or semi-solid supports.

Suitable components further include such groups which have a hydrophilic character. Zwitterionic groups are also comprised.

25

In particular, the dendritic core A may comprises one or more of

$-\text{NH}(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{C}(\text{O})-)_2-$,

$-\text{NH}(\text{CH}_2)_2\text{NHC}(\text{O})\text{CH}_2(\text{N}(\text{CH}_2\text{CH}_2)\text{N})\text{CH}_2\text{C}(\text{O})-$

30 $((-\text{NH}(\text{CH}_2)_3(\text{O})(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{COOH})\text{CH}_2\text{C}(\text{O})-)_q,$

wherein q is as defined above,

$-\text{NH}(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{COOH})\text{CH}_2\text{C}(\text{O})-$

$-\text{NH}(\text{CH}_3)_3(\text{OCH}_2\text{CH}_2)_q\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{COOH})\text{CH}_2\text{C}(\text{O})-$

$-\text{NH}(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{NHC}(\text{O})\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2)_q\text{CH}_2\text{C}(\text{O})\text{NH}-$

35 $(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{COOH})\text{CH}_2\text{C}(\text{O})-.$

In a particular embodiment, the dendritic core comprise one or more of the layer compounds ALL, DAAL, TAAL, PEG, PEG34, and PIP cf. the abbreviations below.

5 In certain applications, it may be advantageous that the dendritic core A is composed of identical units forming successive layers having the same basic structure. In
10 orther, it may be advantageous that the dendritic core A is composed of different units forming layers having an alternating structure.

Each layer of the dendritic core A may suitably be bifocated or trifocated. By this is meant that each layer has two or three possible branching points. Two branching
15 points can be provided by trivalent nitrogen atoms, and three branching points can be provided by tetravalent carbon atoms.

20 In accordance with the definition above, the dendritic core A comprises at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein A comprises at least one ether group. Thus, the dendrimer is not composed entirely of naturally occurring amino acids. However, in
25 some applications, it may be an advantage that the dendritic core A comprises naturally or non-naturally occurring amino acids.

30 The loose structure of the dendrimers of the present invention can be achieved by forming a dendritic core A such that the interdistance between each of the Entity1's and/or each of the Entity2's or visa versa is at least 5, at least 10, at least 20, at least 30, at least 35, at least 40, at least 50, at least 60, at least 70, at least
35 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150, at least

200, at least 210, at least 220, at least 230, at least 250, at least 260, at least 270, at least 280, at least 290, at least 300, at least 310, at least 320, at least 330, at least 340, at least 350, at least 360, at least 5 370, at least 380, at least 390, at least 400, at least 410, at least 420, at least 430, at least 440, at least 450, at least 460, at least 470, at least 480, at least 490, at least 500, at least 525, at least 550, at least 575, or at least 600 bonds. In some embodiments, the 10 interdistance is 35, 770, 130 and 250 bonds, cf. the Examples. By the expression "interdistance" is meant the number of bonds between two entities being attached to same branching point, however, without bonds to other branchings.

15

As appears from the above, the present invention relates to dendrimers, wherein

x1 and x2 are both 0,
x1 is 0, and x2 is 1-1200,
20 x1 is 1-1200, and x2 is 0, and
x1 is 1-1200, and x2 is 1-1200.

The dendrimers of the present invention can be constructed so as to present any combination of Entity1's 25 and Entity2's. Particular interesting possibilities are

- (1) the Entity1's are probes and the Entity2's are labelling compounds,
- (2) the Entity1's are labelling compounds and the 30 Entity2's are probes,
- (3) both the Entity1's and the Entity2's are probes,
- (4) both the Entity1's and the Entity2's are labelling compounds,
- (5) the Entity1's are probes substituted by labelling 35 compounds, and the Entity2's are labelling compounds,

(6) the Entity1's are labelling compounds, and the Entity2's are probes substituted by labelling compound,

(7) one or more of the Entity1's are probes, and the remaining Entity1's are another of the possible

5 substituents as defined above, and the Entity2's are labelling compounds or probes substituted by labelling compounds,

(8) one or more of the Entity2's are probes, and the remaining Entity2's are another of the possible

10 substituents as defined above, and the Entity1's are labelling compounds or probes substituted by labelling compounds,

(9) the Entity1's are the same or different labelling compounds or the same or different probes substituted by

15 the same or different labelling compounds,

(10) the Entity2's are the same or different labelling compounds or the same or different probes substituted by the same or different labelling compounds,

(11) only Entity1's are present (x_1 being 0),

20 (12) only Entity2's are present (x_2 being 0),
as well as protected forms of such dendrimers.

As clearly indicated, the dendrimers of the present invention enable multi-colour presentation (or multi-signal presentation) by choosing the probes/labelling compounds of all or only some the Entity1's and/or all or only some of the Entity2's in accordance with such wish. Thus, it may be possible to have one, two, three, four or even more different labelling. Also, it may be possible

25 to have one, two, three, four or even many more different probes targeting different or same regions.

When a multi-colour option is desired, the following labelling compounds can suitably be used

30 fluorescein, and rhodamine,

35 fluorescein and Cy3,

fluorescein and lissamine,
fluorescein and coumarin,
rhodamine and coumarin,
lissamine and coumarin,
5 fluorescein, rhodamine, and coumarin,
fluorescein, lissamine, and coumarin.

In accordance herewith, dendrimers of the present invention

10

wherein both of the Entity1's and Entity2's are labelling compounds or probes substituted by labelling compounds, wherein at least two, at least three, or at least four of the labelling compounds are different labelling 15 compounds,

15

wherein the Entity1's are a labelling compound or a probe substituted by a labelling compound, and the Entity2's are another labelling compound or a probe substituted by said another labelling compound,

20

wherein both of the Entity1's and Entity2's are probes which may target different or same regions of the same or different targets,

wherein only the Entity1's are present,

wherein only the Entity2's are present,

25

as well as protected forms thereof,

are aspects of the present invention.

30

In the present context, the term "labelling compound" refers to a substituent which is useful for detection, i.e. suitable for generating a visible or otherwise detectable signal directly or indirectly. In accordance with the present invention, suitable labelling compounds comprise fluorophores, biotin, dinitro phenyl radicals, 35 digoxigenin, radioisotope labels such as covalently bound radioisotope labels and complex bound radioactive ions,

enzyme labels, dyes, chemiluminiscence labels, electroluminiscence labels, hapten, antigen or antibody labels, and spin labels. Examples of particular interesting labelling compounds are biotin, fluorescent labels, such as fluorescein labels, e.g. 5-(and 6)-carboxyfluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid and fluorescein isothiocyanate, dinitro phenyl radical, rhodamine, tetramethylrhodamine, cyanine dyes such as Cy2, Cy3 and Cy5, optionally substituted coumarin, R-phycoerythrin, allophycoerythrin, Texas Red and Princeton Red as well as conjugates of R-phycoerythrin and, e.g. Cy5 or Texas Red. Such labelling compounds may optionally be protected by one or more protecting groups.

15 Examples of such protecting groups are given above.

In particular, the probe may be selected from peptide nucleic acids, RNA sequences or DNA sequences or analogues thereof, antibodies, antigens, proteins, peptides or derivatives thereof, epitopes, and biotin, or a protected form thereof.

In accordance with the definitions, the dendrimers of the present invention may thus have up to 2400 (x_1 and x_2 each being 1200) entities (Entity1's and Entity2's) attached thereto. The total number of entities (Entity1's and Entity2's) may suitably be up to 2000, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 50, or 25. The number of Entity1's and Entity2's may be the same or different.

25 In particular, the outermost layer of the dendrimer may have 4, 8, 16, 32, 64, 128, 256, 512, 1024, or 1048 entities attached thereto.

30 In a particular embodiment, the present invention relates to dendrimers having the general formula

(Entity1)_{x1}-A-(Entity2)_{x2} (I)

wherein the dendritic core A is as defined above, and wherein x1 and x2 independently is 2^m, m being an integer of from 1 to 10. In particular, m may be 2, 3, 4 or 5. Thus, m being 2 means that x1 and/or x2 is 4, m being 3 means that x1 and/or x2 is 8, m being 4 means that x1 and/or x2 is 16, or m being 5 means that x1 and/or x2 is 32.

10

In a special embodiment, the present invention relates to dendrimers, wherein the dendritic core A comprises one or more moieties of the structure (Ia)

15

Z[(Z)_z(\square)_y]_z (Ia)

wherein each Z is a group which contains at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein the 20 moiety of formula (Ia) comprises at least one ether group, and wherein each y independently is 2 or 3, and z is an integer of from 1 to 10, with the proviso that y^z ≤ 1200, and protected forms thereof. Each Z group may be any group which provide spacing between the entities on 25 the outermost layer of the dendrimer.

Within the scope of the present invention fall dendrimers, wherein Z groups contribute to the structure of the dendritic core A, wherein

30 x1 and x2 both a 0,
x1 is 0, and x2 is 1-1200,
x1 is 1-1200, and x2 is 0, and
x1 is 1-1200, and x2 is 1-1200,
as well as protected forms thereof.

An important embodiment is the dendrimers terminating in one or more groups (in principle x_1 and/or x_2 other groups) as described above.

5 In a special embodiment, the dendrimers of the present invention may be such, wherein the dendritic core A comprises at least two moieties of the structure (Ia) connected to a naturally or non-naturally amino acid, a peptide nucleic acid moiety, an LNA, a peptide, a 10 protein, an antibody, an antigen, an immune complex, a DNA sequence or an analogue thereof, a RNA sequence or an analogue thereof, a macromolecule, and/or a solid or semi-solid support. A more detailed definition of these terms is given above. Accordingly, dendrimers are formed 15 wherein each of the moieties (Ia) are connected to other compounds, thus forming the dendritic core A. These other compounds may suitably have connected thereto at least 2 moieties of the structure (Ia). However, in certain applications, many moieties of the structure (Ia) may be 20 connected, such as from 2-100, 2-80, 2-70, 2-60, 2-50, 2-35, 2-30, 2-25, 2-20, 2-18, 2-15, 2-12, 2-10, 2-8, 2-5, or 3 or 4. The moieties of the structure (Ia) may be connected terminally or internally to the other compounds 25 as defined hereabove (e.g. alkyl groups, alkenyl groups, alkynyl groups, said groups containing substituents as defined above, amino acids, peptide nucleic acids, LNAs, peptides, proteins, and macromolecules).

In particular, each Z forming the moiety of the structure 30 (Ia) and contributing to the dendritic core A may comprise one or more of C_{1-100} alkyl groups, C_{2-100} alkenyl groups, C_{2-100} alkynyl groups, said alkyl, alkenyl, and alkynyl groups optionally containing one or more functional groups and/or one or more heteroatoms, 35 naturally or non-naturally amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies,

antigens, immune complexes, DNA sequences and analogues thereof, RNA sequences and analogues thereof, and macromolecules.

5 The alkyl, alkenyl and alkynyl groups may in particular be such with C₅₋₈₀, C₅₋₇₀, C₅₋₆₀, C₁₀₋₅₀, C₁₀₋₂₅, C₁₅₋₂₅, and C₁₀₋₂₀ carbon atoms which further optionally contains one or more functional groups and/or one or more heteroatoms. The terms "functional groups" and "heteroatoms" are
10 defined above.

In particular, each Z forming the moiety of the structure (Ia) and contributing to the dendritic core A may comprise one or more of C₁₅₋₂₅ alkyl groups, C₁₅₋₂₅ alkenyl groups, of C₁₅₋₂₅ alkynyl groups, said alkyl, alkenyl, and alkynyl groups optionally one or more functional groups and/or one or more heteroatoms.
15

In a special embodiment, each Z contributing to the dendritic core A may comprise one or more of
20
-NH((CH₂)_qO)_q(CH₂)_qN((CH₂)_qC(O)-)₂,
((-CH₂)_qO)_q(CH₂)_qN((CH₂)_qO-)₂,
((-CH₂)_qO)_q(CH₂)_qC((CH₂)_qO-)₃, and
-NH((CH₂)_qO)_q(CH₂)_qC((CH₂)_qC(O)-)₃,
25

wherein each q independently is 0 an integer from 1 to 8, preferably from 1 to 3. Also comprised is successive layers containing one or more of these specified groups as well as one or more of naturally or non-naturally 30 amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA sequences or analogues thereof, macromolecules, and solid or semi-solid supports, and further layers containing one 35 or more of these specified groups and other layers containing one or more of naturally or non-naturally

amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA sequences or analogues thereof, macromolecules, and solid 5 or semi-solid supports.

In particular, each Z group forming the moiety of the structure (Ia) and contributing to the dendritic core A may comprise one or more of

10 -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-,
-NH(CH₂)₂NHC(O)CH₂(N(CH₂CH₂)N)CH₂C(O)-
(-NH(CH₂)₃(O)(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂COOH)CH₂C(O))_q,
wherein q is as defined above,
-NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(COOH)CH₂C(O)-
15 -NH(CH₃)₃(OCH₂CH₂)_qO(CH₂)₃N(CH₂COOH)CH₂C(O)-
-NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂O(CH₂CH₂)_qCH₂C(O)NH-
(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂COOH)CH₂C(O)-.

In a particular embodiment, the dendritic core comprise 20 one or more of the layer compounds ALL, DAAL, TAAL, PEG, PEG34, and PIP.

In particular, the Z groups contributing to the dendritic core A may be chosen so that the interdistance 25 between each of the Entity1 and/or each of the Entity2 or visa versa or visa versa is at least 5, at least 10, at least 20, at least 30, at least 35, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, 30 at least 140, at least 150, at least 200, at least 210, at least 220, at least 230, at least 250, at least 260, at least 270, at least 280, at least 290, at least 300, at least 310, at least 320, at least 330, at least 340, at least 350, at least 360, at least 370, at least 380, 35 at least 390, at least 400, at least 410, at least 420, at least 430, at least 440, at least 450, at least 460,

at least 470, at least 480, at least 490, at least 500, at least 525, at least 550, at least 575, or at least 600 bonds. In some embodiments, the interdistance is 35, 770, 130 and 250 bonds, cf. the Examples.

5

The dendrimers of the present invention, wherein Z groups contributes to the dendritic core A, can be constructed so as to present any combination of Entity1's and Entity2's. Particular interesting possibilities are

10

(1) the Entity1's are probes and the Entity2's are labelling compounds,

(2) the Entity1's are labelling compounds and the Entity2's are probes,

15 (3) both the Entity1's and the Entity2's are probes,

(4) both the Entity1's and the Entity2's are labelling compounds,

(5) the Entity1's are probes substituted by labelling compounds, and the Entity2's are labelling compounds,

20 (6) the Entity1's are labelling compounds, and the Entity2's are probes substituted by labelling compound,

(7) one or more of the Entity1's are probes, and the remaining Entity1's are another of the possible substituents as defined above, and the Entity2's are

25 labelling compounds or probes substituted by labelling compounds,

(8) one or more of the Entity2's are probes, and the remaining Entity2's are another of the possible substituents as defined above, and the Entity1's are

30 labelling compounds or probes substituted by labelling compounds,

(9) the Entity1's are the same or different labelling compounds or the same or different probes substituted by the same or different labelling compounds,

- (10) the Entity2's are the same or different labelling compounds or the same or different probes substituted by the same or different labelling compounds,
- (11) only the Entity1's are present (x1 being 0),
- 5 (12) only the Entity2's are present (x2 being 0),
as well as protected forms thereof.

Of course one or more of the entities can be replaced by e.g. a reactive group, a free group, or an activated 10 group, thereby terminating the dendritic core.

Thus, an aspect of the dendrimers wherein Z groups contributes to the dendritic core A include those

- 15 wherein one of the Entity1's and the Entity2's is a probe and the other is a labelling compound,
- wherein both of the Entity1's and Entity2's are labelling compounds or probes substituted by labelling compounds,
- wherein at least two, at least three, or at least four of 20 the labelling compounds are different labelling compounds,
- wherein the Entity1's are a labelling compound or a probe substituted by a labelling compound, and the Entity2's are another labelling compound or a probe substituted by 25 said another labelling compound,
- wherein both of the Entity1's and the Entity2's are probes,
- wherein both of the Entity1's and Entity2's are probes which may target different or same regions of the same or 30 different targets,
- wherein only the Entity1's are present, and
- wherein only the Entity2's are present.

35 The labelling compound is preferably selected from fluorophores, biotin, dinitro phenyl radical, digoxigenin, radioisotope labels, or enzyme labels, dyes,

chemiluminiscence labels, haptens, antigen or antibody labels, and spin labels. Examples of such labelling compounds are given above. The labelling compound may be protected by a protecting group. Examples of suitable 5 protecting groups are given above.

In particular, the probe may be selected from peptide nucleic acids, RNA sequences or DNA sequences or analogues thereof, antibodies, antigens, proteins, 10 peptides or derivatives thereof, epitopes, and biotin.

Dendrimers of the present invention wherein Z groups contribute to the dendritic core A are suitably those wherein x_1 and x_2 independently is 2^m , and wherein m is 15 an integer of from 1 to 10. In particular, m may be 2, 3, 4 or 5.

In general, in all the dendrimers of the present invention the Entity1's and Entity2's may be connected to 20 terminally or internally to the substituents in question. Accordingly, at least one of the Entity1's and the Entity2's is connected to a naturally or non-naturally amino acid terminally or internally, a peptide nucleic acid terminally or internally, a peptide terminally or 25 internally, an LNA terminally or internally, a protein terminally or internally, an antibody, an antigen, an immune complex, a RNA sequence or an analogues thereof terminally or internally, a DNA sequence or an analogue thereof terminally or internally, a macromolecule 30 terminally or internally, or a solid or semi-solid support.

In a further aspect, the present invention relates to protected dendrimers as described herein.

The present invention also relates to dendrimer complexes comprising at least one dendrimer as defined herein connected to a naturally or non-naturally amino acid terminally or internally, a peptide nucleic acid 5 terminally or internally, an LNA terminally or internally, a peptide terminally or internally, a protein terminally or internally, an antibody, an antigen, an immune complex, a RNA sequence or an analogue thereof terminally or internally, a DNA sequence or an analogue 10 terminally or internally, a macromolecule terminally or internally, or a solid or semi-solid support, and protected forms thereof.

In particular, the dendrimer complex is such, wherein the 15 dendrimer is connected via at least one of the Entity1's and/or the Entity2's.

Another possibility is that in the dendrimer complex, the 20 dendrimer is connected via a group or groups of the dendritic core A.

The dendrimer complexes of the invention may suitably have 1-100, 1-80, 1-70, 1-60, 1-50, 1-35, 1-30, 1-25, 1-20, 1-18, 1-15, 1-12, 1-10, 1-8, 1-5, or 2, 3 or 4 25 dendrimers as defined herein connected thereto.

In a special embodiment, the dendrimer is internally connected to a naturally or non-naturally occurring amino acid, a peptide nucleic acid, an LNA molecule, a protein, 30 a RNA sequence or a DNA sequence, a macromolecule, or a solid or a semi-solid support.

For example, when the dendrimer complex comprises a DNA or RNA sequence, a peptide nucleic acid, or a LNA 35 molecule, one dendrimer as defined herein may be

incorporated for each 50, 40, 35, 30, 25, 20, 15, 10, 5 amino acid.

5 In accordance with the above, non-limiting examples of the structures of interesting dendrimers/dendrimer complexes of the invention are

10 (1) (labelling compound)_{x1}-dendritic core-(peptide nucleic acid, DNA, protein, and/or antibody connected to one or more alkyl, alkenyl, or alkynyl groups optionally containing functionalities/heteroatoms as defined above)-dendritic core-(labelling compound)_{x2}.

15 In these types of compounds, Entity1's and Entity2's are labelling compounds, and the dendritic core A comprises as part thereof one ore more peptide nucleic acids, DNAs, proteins, and/or antibodies. The alkyl, alkenyl and alkynyl groups may be connected terminally or internally.

20 (2) (labelling compound)_{x1}-dendritic core-(peptide nucleic acid, DNA, protein, and/or antibody)-dendritic core-(labelling compound)_{x2}.

25 In these types of compounds, Entity1's and Entity2's are labelling compounds, and the dendritic core A comprises as part thereof one ore more peptide nucleic acids, DNAs, proteins, and/or antibodies.

(3) (labelling compound)_{x1}-moiety of the structure (Ia)-(peptide nucleic acid, DNA, protein, and/or antibody)-moiety of the structure-(labelling compound)_{x2}.

30 In these types of compounds, Entity1's and Entity2's are labelling compounds, and the dendritic core A comprises moieties of the structure (Ia) connected to one ore more peptide nucleic acids, DNAs, proteins, and/or antibodies.

35 (4) (labelling compound)_{x1}-moiety of the structure (Ia)-(peptide nucleic acid, DNA, protein, and/or antibody

connected to one or more alkyl, alkenyl, or alkynyl groups optionally containing functionalities/heteroatoms as defined above)-moiety of the structure-(labelling compound)_{x2}.

5 In these types of compounds, Entity1's and Entity2's are labelling compounds, and the dendritic core A comprises moieties of the structure (Ia) connected to one or more peptide nucleic acids, DNAs, proteins, and/or antibodies. The alkyl, alkenyl and alkynyl groups may be connected
10 terminally or internally.

(5) (labelling compound)_{x1}-dendritic core-(peptide nucleic acid, DNA, protein, and/or antibody connected to one or more alkyl groups, alkenyl groups, or alkenyl
15 groups having substituents/functional groups as described above)-dendritic core-(labelling compound)_{x2}.

In these types of compounds, Entity1's and Entity2's are labelling compounds, and the dendritic core A comprises as part thereof one or more peptide nucleic acids, DNAs,
20 proteins, and/or antibodies. The alkyl, alkenyl and alkynyl groups may be connected terminally or internally.

(6) (labelling compound)_{x1}-moiety of the structure (Ia)-(peptide nucleic acid, DNA, protein, and/or antibody
25 connected to one or more alkyl groups, alkenyl groups, or alkenyl groups having substituents/functional groups as described above)-moiety of the structure (Ia)-(labelling compound)_{x2}.

In these types of compounds, Entity1's and Entity2's are
30 labelling compounds, and the dendritic core A comprises moieties of the structure (Ia) connected to one or more peptide nucleic acids, DNAs, proteins, and/or antibodies.

(7) (protein or antibody)-dendritic core-(labelling
35 compound)_{x2}.

In these types of compounds, the Entityl's are a protein such as e.g. streptavidin or an antibody, and x_1 is 1. The protein or antibody may be in the form of a monomer, dimer, trimer, tetramer or another multiplier.

5

(8) (protein or antibody)-dendritic core-(labelling compound) $_{x_2}$.

In these types of compounds, the Entityl's are a protein such as e.g. streptavidin or an antibody, and x_1 is 1.

10 The dendritic core A comprises one or more of the moieties of the structure (Ia). The protein or antibody may be in the form of a monomer, dimer, trimer, tetramer or another multiplier.

15 (9) (protein or antibody)-[dendritic core-(labelling compound) $_{x_2}$] $_{1,2,3,\dots}$.

In these types of compounds, the Entityl's are a protein such as e.g. streptavidin or an antibody, and x_1 is 1.

20 The protein or antibody may be in the form of a monomer, dimer, trimer, tetramer or another multiper. As indicated, these types of compounds may have one or more of the [dendritic core-(labelling compound) $_{x_2}$] attached thereto (dendrimer complex).

25 (10) (protein or antibody)-[dendritic core-(labelling compound) $_{x_2}$] $_{1,2,3,\dots}$.

In these types of compounds, the Entityl's are a protein such as e.g. streptavidin or an antibody, and x_1 is 1.

30 The protein or antibody may be in the form of a monomer, dimer, trimer, tetramer or another multiper. The dendritic core A comprises one or more of the moieties of the structure (Ia). As indicated, these types of compounds may have one or more of the [dendritic core-(labelling compound) $_{x_2}$] attached thereto (dendrimer complex).

(11) dendritic core-(labelling compound)_{x2}.

In these types of compounds, x1 is 0. One or more of these dendrimers may be attached to a DNA, a peptide nucleic acid or an antibody via the dendritic core.

5

(12) dendritic core-(labelling compound)_{x2}.

In these types of compounds, x1 is 0. The dendritic core A comprises one or more of the moieties of the structure (Ia). One or more of these dendrimers may be attached to 10 a DNA, a peptide nucleic acid or an antibody via the dendritic core.

(13) (labelling compound attached to peptide nucleic acid, DNA, and/or protein)_{x1}-dendritic core-(labelling 15 compound)_{x2}.

In these types of compounds, the labelling compounds may suitably be different, thus providing a multi-colour signal option.

20 (14) (labelling compound attached to peptide nucleic acid, DNA, and/or protein)_{x1}-dendritic core-(labelling compound)_{x2}.

The dendritic core A comprises one or more of the moieties of the structure (Ia). In these types of 25 compounds, the labelling compounds may suitably be different, thus providing a multi-colour signal option.

(15) (labelling compound attached to peptide nucleic acid, DNA, and/or protein)_{x1}-dendritic core-(peptide, 30 protein, peptide nucleic acid, and/or DNA)_{x2}.

In these types of compounds, the labelling compounds may suitably be different, thus providing a multi-colour signal option.

(16) (labelling compound attached to peptide nucleic acid, DNA, and/or protein)_x-dendritic core-(peptide, protein, peptide nucleic acid, and/or DNA)_{x2}.

In these types of compounds, the labelling compounds may 5 suitably be different, thus providing a multi-colour signal option. The dendritic core A comprises one or more of the moieties of the structure (Ia).

(17) The dendrimer complex may suitably comprise a 10 protein such as tetrameric streptavidin or a dextran macromolecule having connected thereto several dendrimers as defined herein. The dendrimers may each suitably have attached thereto up to 4, 8, 16 or even 32 labelling compounds like fluorescein.

15 (18) The dendrimer complex may suitably comprise a protein such as tetrameric streptavidin or a dextran macromolecule having connected thereto several dendrimers as defined herein. The dendrimers may each suitably have 20 attached thereto up to 4, 8, 16 or even 32 labelling compounds, wherein at least two of them are different, e.g. fluorescein and rhodamine.

25 The protected form of the above embodiments may be very interesting dendrimers and dendrimer complexes. Suitable protecting groups providing the protected dendrimer or dendrimer complex are those indicated above, in particular Boc, Fmoc, and Obz.

30 Although it is not specified in the all embodiments above, the labelling compounds may be the same or be different. Likewise, the probes may be the same or be different. Furthermore, the expression "a probe" is, as mentioned above, intended to include one or more of the 35 possibilities mentioned under the definition of suitable probes, i.e. each Entity1 and/or Entity2 may comprise a

combination of such probes. Likewise, the expression "a labelling compound" may comprise a combination of labelling compounds.

5 Protected dendrimer complexes as described herein also lie within the scope of the present invention.

As mentioned above, the application of the dendrimers and dendrimer/complexes are numerous.

10 Thus, in a further aspect, the present invention relates to the use of the dendrimers and dendrimer complexes as defined herein for detecting the presence of nucleic acid sequences, antibodies, antigens, immune complexes, 15 proteins, or peptides in a sample.

The sample may be any sample to be tested, in particular a blood sample, a bone marrow sample, a chromosome spread, a tissue sample, a tissue section, a cell smear, 20 a biopsy, an organ, a swap, a suspension of cells or parts thereof, and whole cells or parts thereof. The dendrimers are suitable for in vitro as well as in vivo diagnostic.

25 The dendrimers and dendrimer complexes of the invention are suitable for use in almost any assay system. Non-limiting examples are ELISA-based systems, dot blot, flow cytometry, in situ-based assays like ISH, other staining-based assays, other bead- or particle-based assays like 30 turbidimetry, and slide-based systems.

A single dendrimer or dendrimer complex optionally displaying multi-functionalities may be used. Also several dendrimers or dendrimer complexes may be used. Or 35 even a combination of dendrimers and dendrimer complexes may be used.

The dendrimers and dendrimer complexes of the present invention is as mentioned above suitable for the determination of substances present in biological 5 samples. Non-limiting examples of application are given below.

1. For instance, Entity1 may be a probe capable of binding to specific nucleic acid sequences, immune complexes, antibodies, antigens or peptides in a sample, and Entity2 may be a labelling compound or a probe substituted by a labelling compound. This allows a strong enhancement of the signal.
- 15 2. Entity1 may be a probe capable of binding to specific nucleic acid sequences, immune complexes, antibodies, antigens or peptides in a sample, and one or more of the Entity2's may be a probe (e.g. DNA, peptide nucleic acid, or peptide) not binding to the substance in interest in 20 the sample. Another dendrimer/dendrimer complex, wherein Entity1 is a probe binding specifically to Entity2, may then be applied. In this other dendrimer, Entity2 is suitably a labelling compound or a probe substituted by a labelling compound. A third or fourth dendrimer/dendrimer 25 complex can of course be constructed in the same way. In this way, a multi-layer detection system is obtained. A strong enhancement of the signal is the result.
- 30 3. In particular when the dendrimer/dendrimer complex is used for detecting substances present in a low number of copies or even only a single copy, two or more dendrimers may be used. A dendrimer/dendrimer complex wherein Entity1 binds to a specific nucleic acid sequence in a sample, and a dendrimer wherein Entity1 is binding to 35 another specific nucleic acid sequence in a sample in the vicinity of the first sequence, may be applied. The

Entity2's may then be labelling compounds or probes substituted by labelling compound. Of course, additional dendrimers/dendrimer complexes may be constructed along the same pattern. This yields a strong amplification of 5 the observed signal.

4. The principle of 3 may be extended to a multi-layer system as described above under 2.

10 5. The dendrimers/dendrimer complexes are particularly suitable for simultaneous detection of the sense and anti-sense strands of DNA. Entity1 may be chosen so as to bind to nucleic acid sequences of the sense strand, and Entity2 may be chosen so as to bind to nucleic acid 15 sequences of the anti-sense strand. One or more of the Entity1 and/or Entity2 groups may be chosen so as to be labelling compound or probes substituted by labelling compounds making detection possible. Alternatively, a multi-layer system may be constructed in the same way as 20 described above, i.e. one or more of Entity1's and/or Entity2's being probes not binding to the substance of relevance in the sample. This could e.g. be a different DNA, a peptide nucleic acid, a peptide, an antibody, or an antigen.

25 6. The dendrimer/dendrimer complexes may be conjugated to particles via Entity1's/Entity2's. One ore more Entity1's/Entity2's may then be a probe and the remaining labelling compounds.

30 7. The dendrimer/dendrimer complexes may contain labels in the dendritic core (which is possible if the dendritic core contains functional groups). Likewise, Entity1's/Entity2's may be labelled internally via 35 functional groups in the probe.

8. Two or more Entity2's may be directed to sequences located nearby each other. Entity1's may then suitably be labelling compounds.

5 9. The dendrimer/dendrimer complex may suitably comprise one or more nucleic acid sequences and/or peptide nucleic acid moieties within the dendritic core.

10 10. The dendrimer complex may suitably comprise a protein such as tetrameric streptavidin or a dextran macromolecule having connected thereto several dendrimers as defined herein. The dendrimer complexes may each suitably have attached thereto up to 4, 8, 16 or even 32 labelling compounds like fluoresceine. Due to the loose structure of the dendrimers, no or little quenching occurs, and furthermore, the dendrimer/dendrimer complex still has good solubility. This of course yields a strong enhancement of the signal.

20 11. The dendrimer complex may suitably comprise a DNA strand having attached thereto a number of dendrimers. For instance, a dendrimer may be incorporated for each 10-15 base. If the dendrimers comprises labelling compounds, this provides a strong enhancement of the 25 signal/amplification.

Another aspect of the present invention is detection systems comprising at least one dendrimer and/or at least one dendrimer complex as defined herein.

30 In a further aspect, the present invention relates to signal amplification systems comprising at least one dendrimer and/or at least one dendrimer complex as defined herein.

Accordingly, the present invention also relates to use of dendrimers and dendrimer complexes as defined herein as a detection system, as well as the use of dendrimers and a dendrimer complexes as defined herein as a signal 5 amplification system.

Such detection systems or amplification systems are suitable for detecting the presence of nucleic acid sequences, antibodies, antigens, immune complexes, 10 proteins, or peptides in a sample.

Furthermore, the dendrimers and dendrimer complexes of the present invention may be used in various labelling reactions. Thus, the present invention relates to the use 15 of the dendrimers and dendrimer complexes as defined herein for labelling a compound. In particular, the compound to be labelled is selected from peptide nucleic acids, LNAs, RNA sequences, DNA sequences or analogues thereof, antibodies, antigens, immune complexes, 20 proteins, peptides or derivatives thereof, epitopes, streptavidin, and biotin.

For the purpose of labelling reaction, the dendrimers and dendrimer complexes may suitably comprise one or more 25 labelling compounds like fluorophores, biotin, dinitro phenyl radicals, digoxigenin, radioisotope labels, enzyme labels, dyes, chemiluminiscence labels, haptens, antigen or antibody labels, and spin labels.

30 In a further aspect, the present invention relates to labelling kits for labelling a compound, which kits comprise one or more dendrimers and/or dendrimer complexes. In an embodiment of such kit, the kit further comprises a labelling compound. In another embodiment, 35 the kit is such, wherein the dendrimer or dendrimer complex comprises a labelling compound. The labelling

compound may suitably be selected from fluorophores, biotin, dinitro phenyl radicals, digoxigenin, radioisotope labels, enzyme labels, dyes, chemiluminiscence labels, haptens, antigen or antibody labels, and spin labels.

It is also an object of the present invention to provide detection kits for detecting a compound, which kits comprise one or more dendrimers and/or one or more dendrimer complexes as defined herein, wherein the dendrimer and/or the dendrimer complex comprise(s) one or more labelling compounds. Of course, the dendrimers/dendrimer complexes can be unlabelled and in that case the kit may include means for labelling the dendrimers/dendrimer complexes. Suitable labelling compounds/means for labelling include fluorophores, biotin, dinitro phenyl radicals, digoxigenin, radioisotope labels, enzyme labels, dyes, chemiluminiscence labels, haptens, antigen or antibody labels, and spin labels.

The dendrimers of the present invention can be prepared by various methods, e.g. such used in connection with peptide synthesis. The person skilled in the art will readily know how to apply solid phase and liquid phase synthetic procedures when such are needed.

In particular, the building blocks of the dendrimer may be synthesised individually. Subsequently, the dendrimer may be assembled. This enables the preparation of the dendrimers as well as the protected forms.

Alternatively, the dendrimer may be synthesised by a step-by-step process, whereby the whole dendrimer is synthesised and the various entities attached in a

last step. This enables the preparation of the dendrimers as well as the protected forms.

5 In yet another process of preparation, the dendrimer can be synthesised step-wise in solution, whereby the product of the reaction is precipitated, and the procedure is repeated until the desired product is obtained.

10 For the synthesis, various solvents, reagents, protecting groups, activating agents etc. are to be applied. A synthetic strategy is outlined below and the reagents etc. mentioned in connection therewith can be applied in any other synthesis regime.

15 Thus, one process for preparing the dendrimers of the formula (I) or protected forms thereof as defined above comprises

20 preparing a dendritic core A by chemical/peptide synthesis, depending on the components of the dendritic core A, which dendritic core A has a number of protecting groups, which can be removed by deprotection, if desired, removing the protecting groups by use of a suitable deprotecting agent,

25 if desired, coupling the Entity1's and/or the Entity2's to the dendritic core A, thereby obtaining the dendrimer.

Another process for preparing the dendrimers of the formula (I) or protected forms thereof comprises

30 preparing building blocks for the dendritic core A by chemical/peptide synthesis, depending on the components of the dendritic core A,

35 assembling the dendritic core A, which dendritic core A has a number of protecting groups, which can be removed by deprotection,

if desired, removing the protecting groups by use of a suitable deprotecting agent,

if desired, coupling the Entity1's and/or the Entity2's to the dendritic core A, thereby obtaining the dendrimer.

5

It is to be understood that the number of protecting groups may correspond to the number of Entity1's and Entity2's (i.e. the number of x_1 and x_2). However, fewer or more protecting groups are envisaged.

10

Yet another process for preparing the dendrimers of the invention or protected forms thereof comprises

coupling a starting material, which may be protected, to 15 a substrate, e.g. a polyethylene glycol compound, which coupling product is soluble,

subjecting the coupling product to deprotection, if necessary, and a coupling reaction in solution,

precipitating the so formed product, and

20 if desired, repeating the above procedures,

thereby obtaining the dendrimer or the protected form thereof.

The protecting groups may be removed in one step or in 25 more steps such as two, depending on whether orthogonal chemistries are used (see below). The reaction may optionally take place in a suitable solvent. The reaction temperature can be adjusted to facilitate the reaction.

30 The dendrimer complexes and the protected forms thereof of the present invention can be assembled by coupling procedures known to the person skilled in the art.

35 Thus, in a further aspect, the present invention relates to dendrimers and dendrimer complexes and protected forms thereof obtainable by the processes described herein.

Non-limiting examples of synthesis and assembly are given below.

5 One synthetic rational is described herebelow. Others are envisaged in the Examples.

A method for the preparation of a dendrimer having the formula (I)

10

(Entity1)_{x1}-A-(Entity2)_{x2} (I)

wherein A is a dendritic core having at least one N-atomic branching point, said branching point not being 15 part of a naturally occurring amino acid, and wherein A comprises at least one ether group, each Entity1 and Entity2 are independently probes which optionally are substituted by one or more labelling compounds, a labelling compound, or a probe having 20 reactive groups,

x1 is 1, and x2 is 0 or an integer of from 1 to 1200, or a protected form thereof

which method comprises the steps of

25

(a) reacting a compound of the formula

Entity1-Z-(Entity2)_y

wherein Z is a group containing at least one branching point, Entity1 and Entity2 are suitable protecting groups 30 which may be identical or different, and x2 is 2 or 3, with a suitable deprotecting agent optionally in a suitable solvent, thereby obtaining a compound of the formula Entity1-Z and a compound of the formula Z-(Entity2)_y, wherein Entity1 and Entity2 are protecting 35 groups,

(b) reacting the compounds obtained in step (a) with each other optionally in a suitable solvent, thereby obtaining a compound of the formula

Entity1-Z[Z-(Entity2)_y]_y (III)

5 wherein Entity1 and Entity2 are protecting groups,

(c) optionally deprotecting the compound obtained in step (b) with a suitable deprotecting agent optionally in a suitable solvent for further reaction, and optionally 10 reacting the resulting compounds with each other or with one of the compounds of step (a) or (b),

(d) obtaining a resulting protected compound which may be deprotected for further reaction with a suitable 15 deprotecting agent optionally in a suitable solvent,

(e) repeating steps (c) and (d) a desired number of times, thereby obtaining a compound of the formula

Entity1-A-(Entity2)_{x2} (IV)

20 wherein Entity1 and Entity2 are protecting groups, x2 is as defined above, and A is a dendritic core having the structure

Z[Z]_z[_y]_z (Ia)

wherein z is as defined above,

25 (f) if desired, removing the protecting groups Entity1 and Entity2 simultaneously or individually with a suitable deprotecting agent optionally in a suitable solvent, and coupling the so obtained deprotected 30 compound with Entity1 and Entity2, thereby obtaining the dendrimer of formula (I).

The dendrimers of the general formula (I)

35 (Entity1)_{x1}-A-(Entity2)_{x2} (I)

wherein A is a dendritic core having at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein A comprises at least one ether group,

5 each Entity1 and Entity2 are independently probes which optionally are substituted by one or more labelling compounds, a labelling compound, or a probe having reactive groups,
x1 and x2 are independently 0 or an integer of from 1 to
10 1200,
and protected forms thereof,

can be prepared by a method comprising the steps of

15 (f1) converting by deprotection one or more compounds of the general formula

Entity1-A-(Entity2)_{x2} (IV)

wherein A and x2 are as defined above, and Entity1 and Entity2 are suitable protecting groups,

20 with a suitable deprotecting agent optionally in a suitable solvent, into one or more compounds of the general formula

A-(Entity2)_{x2},

wherein Entity2 is a protecting groups,

25

(f2) reacting the compound of step (f1) with a compound of the formula A-(Entity1)_{x1}, wherein Entity1 is a protecting group, or with each other, thereby obtaining a compound of the general formula

30 (Entity1)_{x1}-A-(Entity2)_{x2} (V)

wherein x1 and x2 are as defined above, and Entity1 and Entity2 are protecting groups,

35 (f3) if desired, removing the protecting groups Entity1 and Entity2 simultaneously or individually with a suitable deprotecting agent optionally in a suitable

solvent, and coupling the so obtained deprotected compound with Entity1 and Entity2 as defined above, thereby obtaining the dendrimer of formula (I).

5 In the above, the prefix "y" defines whether a given Z group is bifocated (y being 2) or trifocated (y being 3).

Within the present context, "protection groups" is intended to mean groups which can be removed with a 10 suitable deprotection agent. Examples of suitable protection groups are Fmoc, Boc, Mtt, Dde, All, ODMab, Aloc, Otbu, OMe, OBz, Z, MOM, and benzyloxycarbonyl. The choice of protecting group depends i.a. on the type of group which need to be protected. Some of the above- 15 indicated groups are suited for protecting -COOH groups, and others are suited for protecting -NH₂ groups. When two types of protecting groups are used, such are suitably selected so as to be orthogonal protecting groups. Furthermore, protecting groups should be selected 20 so as to be compatible with the various entities on the intermediary compounds and/or the resulting dendrimer.

The term "suitable solvent" is intended to comprise solvents which allow the reaction to take place. Examples 25 of suitable solvents are e.g. water, and organic solvents such as acetone, acetonitrile, cyclohexane, DMSO, DMF, dichloromethane, NMP, DIPEA, benzene, and toluene, as well as mixtures thereof.

30 Within the present context, "suitable deprotecting agent" is intended to mean an agent allowing removal of the protecting groups. The choice of deprotecting agent depends on the choice of protecting groups. Examples of suitable deprotecting agents are TFA, TFMSA, HBr, HCl, 35 HF, piperidine, DBU, hydrazine, base, nucleophiles, Pd/C/H₂, and Pd/AcOH/NMM.

The method of outline above is described in greater detail below.

5 Step (a)

In step (a), a compound of the formula Entity1-Z-(Entity2)_y, wherein Entity1 and Entity2 are protecting groups, (a so-called dendrimer monomer) is reacted with a suitable deprotecting agent. The reaction may or may not 10 take place in a solvent, i.e. the deprotecting agent may be sufficient to provide solubility. Suitable solvents are e.g. water, and organic solvents as defined above as well as mixtures thereof. If the Entity1-side of the Z group is an amine, suitable protecting groups are e.g. 15 Boc, Fmoc, benzyloxycarbonyl, phthalimide, Mtt, Mmt, and trifluoroacetyl. If the Entity1 group is an acid, suitable protecting groups are e.g. benzyl, methyl, tert-butyl, MOM, and silyl esters. The same protecting groups are applicable for Entity2 having regard to whether an 20 amine or an acid group is to be protected. The deprotecting agent(s) is/are selected so as to be compatible with the protection group to be removed. Removal of both deprotection groups can be accomplished at the same time e.g. by strong acid or HBr, or by mixing 25 various deprotecting agents. The deprotection may be carried out at room temperature, under cooling e.g. on dry ice, or at an elevated temperature such as at from 100°C to 180°C. Photochemical deprotection may also be an option.

30

In certain cases, it is advantageous to start out with two different reactions containers, each container containing the starting material, and in one removing the Entity1 protecting groups, and in the other removing the 35 Entity2 protecting groups. Alternatively, the reaction can be carried out in the same reaction container,

selecting the Entity1 and Entity2 protecting groups so as to not being removable by the same deprotecting agent. In either case, a compound of formula Entity1-Z and a compound of formula Z-(Entity2)_y, wherein Entity1 and Entity2 are protecting groups, are obtained.

Removal of the protecting groups imply that the Z group now has functional groups ready for further reaction, the number of functional groups depends on the possible number of branching points. Such functional groups include -COOH, -NH₂, cyclic anhydrides, thiols, alcohols, and halogens.

Two different starting compounds may be used, giving raise to dendrimers wherein the Z groups are mutually different.

Step (b)

In step (b), the compounds obtained in step (a) are reacted with each other, thereby yielding an intermediary compound of the formula (III) Entity1-Z[Z-(Entity2)_y]_y, wherein Entity1 and Entity2 are protecting groups. The reaction may be carried out at room temperature, under cooling e.g. on dry ice, or under heating to a temperature of from 100°C to 180°C. Suitable solvents for the reaction include those mentioned above and mixtures thereof. However, the reaction need not be carried out in a solvent.

The product of this step is a protected dendrimer of formula (I). Upon removal of Entity2 protecting groups and conjugation of Entity2 groups, the dendrimer is formed. If y is 2, the dendrimer has four functionalities. If y is 3, the dendrimer has $2^3 = 6$ functionalities. Some of these may be in the free form, i.e. meaning that some of them do not carry a Entity2

group. If the protecting groups are not removed, a protected dendrimer is obtained.

As in step (a), removal of the protecting groups yields a 5 compound having multiple functional groups which can be subjected to reaction.

The intermediary compound can, however, be subjected to further reaction extending the dendritic core. If this is 10 desired, the process is proceeded according to step (c). Otherwise, the process proceeds according to step (f).

Step (c)

In step (c), the dendritic core is extended further. If 15 further extension is not desired, the compound obtained in step (b) is subjected to reaction according to step (f). Thus, step (c) is an optional step.

The intermediary compound obtained in step (b) having the 20 formula (III) Entity1-Z[Z-(Entity2)_y]_y, wherein Entity1 and Entity2 are protecting groups, is deprotected with a suitable deprotecting agent optionally in a suitable solvent. The deprotecting agent is selected so as to be capable of removing either the Entity1 protecting groups 25 or the Entity 2 protecting groups, or, alternatively, both the Entity1 and the Entity2 protecting groups simultaneously. Accordingly, mixtures of deprotecting agents may be used. Suitable deprotecting agents are those given above.

30 Deprotection yields a compound having multiple functional groups for further reaction. Such functional groups are e.g. -COOH, -NH₂, cyclic anhydride, thiol, alcohols, and halogens.

The deprotection may optionally take place in a suitable solvent. Examples of suitable solvents or mixtures of solvents are as described above (step (a) and (b)). The choice of solvent/solvents depends on the choice of 5 protecting groups and reaction conditions.

The reaction may take place at room temperature, under cooling e.g. on dry ice, or with heating. Photochemical reaction is also possible.

10

The deprotection can be carried out in two reaction containers as described above., one in which Entity1 is removed, yielding a compound of the formula $Z[Z-(Entity2)_y]_y$, and one in which Entity2 is removed, 15 yielding a compound of the formula Entity1- $Z[Z]_y$, wherein Entity1 and Entity2 are protecting groups, and wherein each $[Z]$ has y functional groups available for further reaction. As apparent from the formula, there are y such $[Z]$ groups.

20

The compounds so obtained can be reacted with each other, or can be reacted with one of the compounds of step (a) or (b). Alternatively, the deprotected compound can be reacted with a compound having one or more mutually 25 different Z groups, thus giving raise to dendrimers in which the various Z groups are mutually different.

If the deprotected compound is reacted with a compound in which the Entity1 protecting group is removed, the 30 reaction gives raise to a dendrimers of formula (I) having a plurality of Entity1's and Entity2's upon conjugation with such entities. If the protecting groups are not removed, a protected dendrimer is obtained.

35 If the deprotected compound is reacted with a compound in which the Entity2 protecting group is removed, the

reaction gives raise to dendrimers of formula (I) upon conjugation with Entity1 and Entity2.

Step (d)

5 In step (d), the protected compound obtained in step (c) may be deprotected for further reaction. Deprotection optionally takes place in a suitable solvent. Examples of suitable solvents are those mentioned above under step (a), (b) and (c). Deprotection is carried out using
10 suitable deprotecting agents selected so as to be compatible with the protecting groups to be removed, further having regard to whether different protecting groups are to be removed. Examples of suitable deprotecting agents are given above.

15

Step (e)

In step (e), deprotecting and reaction are repeated a desired number of times until the dendritic core has the desired size. Thus, an intermediary compound of formula 20 (IV) Entity1-A-(Entity2)_{n2}, wherein Entity1 and Entity2 are protecting groups, is obtained.

When one or more dendrimers of the desired form is/are obtained, such can be attached to one or more nucleic acid or DNA sequences and/or peptide nucleic acid 25 moieties, thereby obtaining a dendrimer having such sequences within the dendritic core.

Step (f)

30 In step (f), removal of the Entity1 and Entity2 protecting groups is accomplished. Removal of the Entity1 and Entity2 protecting groups can be carried out at the same time, or in different steps. This depends i.a. on the choice of deprotecting agent and the choice of 35 protecting groups. Suitable deprotecting agents are indicated above. The reaction may or may not take place

in a suitable solvent or mixtures of such solvents. Examples of suitable solvents are given above.

Subsequently, the deprotected intermediary compound is
5 coupled to suitable Entity1's and Entity2's resulting in
a dendrimer of formula (I). Coupling conditions are
selected so as to be compatible with the nature of
Entity1's and Entity2's. Coupling may suitably be carried
out using a suitable coupling agent such as
10 carbodiimides, HBTU, HATU, TBTU, BOP, PyBOP, PyAOP,
DhbtOH esters, Pfp, Bt, and At. Coupling may optionally
take place in a solvent or a mixture of various solvents
such as those mentioned above. Coupling may be carried
out under room temperature, under cooling, under heating,
15 or by means of a photochemical reaction. Coupling may be
accomplished by converting the dendrimer to the activated
form that is very reactive, e.g. an anhydride, a
haloacetyl derivative and other halogenides, thiols,
maleimides, and NHS esters.

20

Step (f1)

In step (f1), an intermediary compound of the formula
(IV) Entity1-A-(Entity2)_{n2}, wherein Entity1 and Entity2
are protecting groups, is deprotected using a suitable
25 deprotecting agent. Suitable deprotecting agents are e.g.
those given above. The reaction may or may not take place
in a suitable solvent or a mixture of such solvents. The
reaction may be performed at room temperature, under
cooling e.g. on dry ice, or with heating to about 100-
30 180°C.

The purpose is to obtain a dendrimer of the formula (I)
which has multiple entity presenting capabilities at both
"sides" of the dendritic core..

35

By removal of the Entity1 protecting groups, a compound of the formula A-(Entity2)_{x2}, wherein Entity2 is a protecting group, is obtained.

5

If a dendritic core of a smaller size is desired, the intermediary compound of formula (III) obtained in step (b) can be subjected to reaction (step (c)) with a compound wherein the Entity1 protecting groups has also 10 been removed. In this way, a protected dendrimer of formula (I) is also obtained, however, having a smaller dendritic core compared to the dendrimer of formula (II) obtained by reaction of the intermediary compound of formula (IV).

15

Step (f2)

In step (f2), the deprotected compound of step (f1) is subjected to reaction with another intermediary compound of formula (IV) in which Prot1 has been removed. 20 Alternatively, the deprotected compound of step (f1) can be subjected to reaction with another compound in which Prot1 has been removed leading to dendrimers in which the Z groups are mutually different.

25 As for the other above-indicated steps, the reaction can take place with or without solvent. The reaction can be performed at room temperature, under cooling or heating, or by photochemical reaction.

30 Suitable deprotecting agents are e.g. those given above.

By this reaction, an intermediary compound of the formula (V), (Entity1)_{x1}-A-(Entity2)_{x2}, wherein Entity1 and Entity2 are protecting groups, is obtained.

35

Step (f3)

In step (f3), the protecting groups are removed. By removal of the protecting groups, functional groups which are reactive are formed (activated groups). Such functional groups are e.g. -COOH, -NH₂, cyclic anhydride, 5 thiols, alcohols, and halogens. Removal of the Entity1 and Entity2 protecting groups can be performed at the same time or in multiple steps.

Subsequently, coupling with Entity1 and Entity2 are 10 performed. Coupling is performed as described above under step (f).

The Entity1 and Entity2 protecting groups may be mutually different or identical. For most purposes, it is most 15 suitable that the Entity1 and Entity2 protecting groups are mutually different since this allows coupling of Entity1's and Entity2's which are mutually different.

In all the above-described steps, it may be necessary to 20 protect sensitive side-chain groups. This can be done using suitable temporary side-chain protecting groups such as Boc, tBu, Z, and Cbz. Side-chain protective groups may be removed by means of an acid such as TFA, TFMSA, HBr, HCl or HF, a base, or a nucleophil like 25 pyridine.

Compared with conventional procedures for obtaining dendritic compounds, the present method is a liquid phase reaction, whereas most conventional procedures are based 30 on solid-phase techniques.

Within the scope of the present invention is also the various intermediary compounds. Furthermore, the present invention also encompasses dendrimers and intermediary 35 obtainable by such manufacturing methods.

The invention is further illustrated by the following, non-limiting examples.

EXAMPLES

5

EXAMPLE 1

Preparation of 1,1-(tert-butoxycarbonyl)-1,15-diaza-4,7,-10-trioxapentadecane; cf. Figure 4, No. 1

10

21.8g, 0.1 mol, (Boc)2O dissolved in dioxane (200 ml) was added drop-wise to 110g, 0.5 mol, 1,13-diamino-4,7,10-trioxatridecane dissolved in dioxane (400 ml). Following 20 minutes stirring after addition and 1 h additional stirring, the solvent was evaporated, and the resulting oil partitioned between water (300 ml) and dichloromethane (300 ml). The organic phase was reduced to an oil and purified by column chromatography on silica. The product eluted (r.f. 0.5) with 10% ammonia saturated methanol in dichloromethane. Yield 23.0 g colourless oil (72%). Mw. calculated for C₁₅H₃₂N₂O₅ 320.3. Found 321.9 (MH⁺, MALDI-TOF MS).

EXAMPLE 2

25

Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis(benzylloxycarbonylmethyl), Figure 4, No. 2

30 Protected dendrimer monomer having the form Entity1-A-(Entity2)₂, dendritic core A = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O-(CH₂)₃N(CH₂C(O))₂-, Entity1 = Boc, Entity2 = OBz, x₁ = 1, x₂ = 2

35 6.4 g, 0.02 mol, 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,-10-trioxapentadecane was dissolved in DMF (25 ml) and

DIPEA (13 ml). 2-bromoacetic acid benzyl ester, 10.0 g, 4.3 mol, was added, and after 5 minutes the reaction mixture was further heated to 80°C for 35 min. The solvents were evaporated, the residue partitioned between 5 saturated NaHCO₃ (500 ml) and ethyl acetate (500 ml). The organic phase was washed with water (2×500 ml), evaporated to dryness and purified on silica, and the product was eluted (r.f. 0.5 with 1:1 ethylacetate/-hexane). Yield 9.9 g pale yellow oil (80%). Mw 10 calculated for C₃₃H₄₈N₂O₉ 616.8. Found 618.2 (MH⁺, MALDI-TOF MS).

EXAMPLE 3

15 Preparation of 3,1-(tert-butoxycarbonyl)-1,15-diaza-4,7,-10-trioxapentadecane-15-diacetic acid, Figure 4, No. 3

Deprotected dendrimer monomer having the form Entity1-A-(free acid)₂, A = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, 20 Entity1 = Boc, x1 = 1, dendritic core terminates in a free acid

3.09 g, 5 mmol, 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,-10-trioxapentadecane-15-bis(benzyloxycarbonylmethyl) was 25 dissolved in methanol (30 ml). 10 % Pd/C (300 mg) was added, and the reaction mixture hydrogenated (deprotection) at room temperature and room pressure for 1 h. Pd/C was spun down, and the supernatant was reduced to dryness. Yield 2.2 g colourless resin (99%). Mw. 30 calculated for C₁₉H₃₆N₂O₉ 436.5. Found 437.9 (MH⁺, MALDI-TOF MS).

EXAMPLE 4

Preparation of 4,1,15-diaza-4,7,10-trioxapentadecane-15-bis(benzyloxycarbonylmethyl) (TFA adduct), Figure 4, No. 4

5 Dendrimer having formula (free amine)-A-(Entity2)₂, A = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity2 = OBz, x₂ = 2, dendritic core terminates in free amine form

10 6.78 g, 11 mmol, 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,- 10-trioxapentadecane-15-bis(benzyloxycarbonylmethyl) was evaporated twice to dryness from TFA (2×20 ml). Yield approximately 13 g of TFA adduct as a pale yellow oil (100%). Mw. calculated for C₂₈H₄₀N₂O₇ 516.5. Found 517.9 (MH⁺, MALDI-TOF MS).

15

EXAMPLE 5

Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)], Figure 4, No. 5

Protected dendrimer having the form Entity1-A-(Entity2)₂, dendritic core A = three -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-N(CH₂C(O))₂- groups, Entity1 = Boc, Entity2 = OBz, x₁ = 1, x₂ = 4

13 g, 11 mmol, 1,15-diaza-4,7,10-trioxapentadecane-15-bis(benzyloxycarbonylmethyl) (TFA adduct) was dissolved 30 in DMF (25 ml) and triethylamine (7.5 g). 2.2 g, 4.9 mmol, 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-diacetic acid in DMF (4 ml) was added, followed by 6.24g, 12 mmol, PyBOP. The mixture was stirred for 2 h at 45°C, the solvents were evaporated. 35 The residue was partitioned between saturated NaHCO₃ (200 ml) and DCM (500 ml). The DCM phase was further extracted

twice with water (2×200 ml) and reduced to dryness. The resulting oil was purified on silica, and eluted (r.f. 0.5) with 15% methanol in ethyl acetate. Yield 3.7 g as a viscous oil (53%). Mw. calculated for C₇₅H₁₁₂N₆O₂₁ 1433.8.

5 Found 1435.1 (MH⁺, MALDI-TOF MS).

EXAMPLE 6

10 Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-diacetic acid], Figure 4, No. 6

15 Partly protected dendrimer of the structure Entity1-A-(free acid)₄ (IV), dendritic core A comprising 3 groups -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity1 = Boc, x₁ = 1, dendritic core terminates in free acid form -COOH

20 1.43 g, 1 mmol, 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,-10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)] was dissolved in methanol (15 ml). 10 % Pd/C (150mg) was added, and the reaction mixture hydrogenated at room temperature and room pressure for 1 h. Pd/C was spun down, and the supernatant reduced to dryness. Yield 1.04 g as a viscous

25 oil (97%). Mw. calculated for C₄₇H₆₈N₆O₂₁ 1073.2. Found 1075.2 (MH⁺, MALDI-TOF MS).

EXAMPLE 7

30 Preparation of 1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)], Figure 4, No. 7

35 Partly protected dendrimer of formula (free amine)-A-(Entity2)₂, dendritic core A comprising 3 moieties of formula -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity2

= OBz, x2 = 4, dendrimer terminates in free amine form -NH₂

7.15 g, 5 mmol, 1-(tert-butoxycarbonyl)-1,15-diaza-
5 4,7,10-Trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)] was evaporated from TFA twice to dryness (2x30 ml). Yield approximately 15 g of TFA adduct as a pale yellow oil (100%). Mw. calculated for C₇₀H₁₀₄N₆O₁₉ 1333.6.

10 Found 1334.1 (MH⁺, MALDI-TOF MS).

EXAMPLE 8

15 Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxa-
pentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxa-
heptadecane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxahepta-
decane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-
17-bis(benzyloxycarbonylmethyl)]], Figure 5, No. 8

20 Protected dendrimer of formula Entity1-A-(Entity2)₈, dendritic core A comprising 15 moieties of formula -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity1 = Boc, Entity2 = OBz, x1 = 1, x2 = 16

25 15 g, 5mmol, 1,15-diaza-4,7,10-trioxapentadecane-15-bis-[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)] (TFA adduct) was dissolved in DMF (30 ml) and triethylamine (9 g). To this solution was added 1.04 g, 0.97 mmol, 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-diacetic acid] followed by 3.12 g, 6 mmol, PyBOP. The mixture was stirred at 45°C for 2 h and the solvents were evaporated. The residue was taken up in DCM (200 ml) and methanol (20 ml) and extracted with saturated NaHCO₃ (200 ml), followed by water (2x50 ml). The organic phase was evaporated to

dryness and purified on silica, eluding (r.f. 0.3) with 15% methanol in DCM. Yield 2.1 g colourless oil (33%). Mw. calculated for $C_{327}H_{496}N_{30}O_{93}$ 6335.7. Found 6333.6 (MH^+ , MALDI-TOF MS).

5

EXAMPLE 9

10 Preparation of 1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)], Figure 5, No. 9

Partly protected dendrimer of formula (free amine)-A-(Entity2)₂, dendritic core A comprising 3 moieties of formula $-NH(CH_2)_3O(CH_2)_2O(CH_2)_2O(CH_2)_3N(CH_2C(O))_2-$, Entity2 = OBz, x2 = 4, dendrimer terminates in free amine form $-NH_2$

15 1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bisbenzyloxycarbonylmethyl] 15 g, (5 mmol of TFA adduct, cf. Example 7) was dissolved in DCM (150 ml) and neutralised with 0.5 M Na₂CO₃ (150 ml). The organic phase was isolated. Toluene (50 ml) was added, and the solvents were evaporated. Yield 5.7 g as a viscous oil (85%). Mw. calculated for C₇₀H₁₀₄N₆O₁₉ 1333.6. Found 1334.9 (MH^+ , MALDI-TOF MS). This compound was observed to undergo slow ammonolysis at room temperature.

20

EXAMPLE 10

30

Preparation of 1-(2-bromoacetyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)], Figure 5, No.

10

35

Activated dendrimer of the form (activated group)-A-(Entity2)₄, activated group = bromoacetyl, Entity2 = OBz, x2 = 4, dendritic core terminates in an activated group

5 1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis-(benzyloxycarbonylmethyl)], 9, 5.7 g, 4.25 mmol, in toluene (25 ml) was added drop-wise over 20 minutes to bromoacetic anhydride 1.3 g, 5 mmol, and lutidine 870 mg, 8 mmol, in ice bath 10 cooled DCM (25 ml). Further DCM (100 ml) was added to the mixture, which was then extracted with NaHCO₃ (100 ml). The organic phase was filtered and evaporated to dryness. The resulting oil was purified on silica, eluding (r.f. 0.5) with 10% methanol in DCM. Yield 5.25 g 15 as a viscous oil (85%). Mw. calculated for C₇₂BrH₁₀₅N₆O₂₀ 1454.7. Found 1456.1 (MH⁺, MALDI-TOF MS).

EXAMPLE 11

20 Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis-[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis-(benzyloxycarbonylmethyl)]), Figure 5, No. 11

25 Protected dendimer of the form Entity1-A-(Entity2)₈, Entity1 = Boc, Entity2 = OBz, x1 = 1, x2 = 8, dendritic core A comprising 7 NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-N(CH₂C(O))₂-

30 1-(2-bromoacetyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis-(benzyloxycarbonylmethyl)], 5.25 g, 3.6 mmol, and 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane, 580 mg 1.8 mmol was dissolved in DMF (6 ml) and DIPEA (1 ml). The mixture was stirred at 80°C for 2 35

hours, and the solvents were removed. The resulting oil was taken up in DCM (100 ml) which was extracted with saturated NaHCO₃ (100 ml) then washed with water (2×20 ml). After solvent removal the product was purified on 5 silica, eluding (r.f. 0.3) with 10% methanol in DCM. Yield 4.5 g as a viscous oil (81%). Mw calculated for C₁₅₉H₂₄₀N₁₄O₄₅ 3067.73. Found 3069.2 (MH⁺, MALDI-TOF).

EXAMPLE 12

10

Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)]], Figure 6, No. 12

15

Partly protected dendrimer of the form (free amine)-A-(Entity2)₁₆, dendritic core A comprising 15 moieties of formula -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity2 20 = OBz, x₂ = 16, x₁ = 0, dendritic core terminates in a free amine

The compound of Example 8 (100 mg) was dissolved in TFA (1 ml) and after 5 minutes, the product could be isolated 25 by precipitation with ether. The yield was assumed to be quantitative. Mw. calculated for C₃₂₂H₄₈₈N₃₀O₉₁ 6235.7. Found 6237.3 (MH⁺, MALDI-TOF MS).

EXAMPLE 13

30

Preparation of 1-(biotinyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(benzyloxycarbonylmethyl)]], Figure 6, No. 13

35

Derivatised dendrimer of the form Entity1-A-(Entity2)₁₆, dendritic core A comprising 15 moieties of formula -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity1 = biotinyl, Entity2 = OBz, x₁ = 1, x₂ = 16

5

The compound of Example 12 (100 mg) was dissolved in NMP (1 ml) and DIPEA (0.1 ml), and biotin (5 mg) was added followed by PyBOP (8 mg). After 1 hour at 45°C, the crude product was isolated by precipitation with ether (8 ml).

10 The so obtained compound was re-dissolved in 10 ml DCM and extracted with NaHCO₃ (10 ml). The organic phase was reduced to dryness to yield the product. Yield 80 mg (79%). Mw. calculated for C₃₃₂H₅₀₂N₃₂O₉₃S 6461.8. Found 6463.2 (MH⁺, MALDI-TOF MS).

15

EXAMPLE 14

20 Preparation of 1-(biotinyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(carboxymethyl))], Figure 6, No. 14

25 Dendrimer of the form Entity1-A-(free acetic acid)₁₆, dendritic core A comprising 15 moieties of formula -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, Entity1 = biotinyl, x₁ = 1, dendrimer terminates in free acetic acids

30 The compound of Example 13 (80 mg) was dissolved in 2 ml methanol and 30 mg Pd/C was added. The mixture was hydrogenated under atmospheric pressure for 1 hour. The catalyst was filtered off, and the mixture reduced to dryness. Yield: 62 mg (100%). Mw. calculated for 35 C₂₂₀H₄₀₆N₃₂O₉₃S 5019.7. Found 5022.3 (MH⁺, MALDI-TOF MS).

EXAMPLE 15

5 Preparation of 1-(biotinyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-16-(2,6-dioxomorpholin-4-yl)]], Figure 7, No. 15

10 Activated dendrimer of the form Entity1-A-(activated group)₈, dendritic core A comprising 17 moieties with 7 -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, and the peripheral 8 groups -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-(2,6-dioxomorpholin-4-yl)-, Entity1 = biotinyl, x₁ = 1, dendrimer terminates in N-2,6-dioxymorpholine

15 The compound of Example 14 (10 mg) was dissolved in pyridine (100 μ l) and diisopropylcarbodiimide (4 μ l) was added. After 30 minutes at 55°C, the product was precipitated by addition of ether. Yield: 9 mg (91%). Mw. 20 calculated for C₂₂₀H₃₉₀N₃₂O₈₅S 4875.7. Found 4876.5 (MH⁺, MALDI-TOF MS).

EXAMPLE 16

25 Final coupling of dendrimer from Example 15, Figure 7, No. 16

30 Dendrimer of formula of the form Entity1-A-(Entity2)₈, dendritic core A comprising 15 groups with 7 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, and the peripheral 8 groups = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-(N(CH₂COOH)-(CH₂C(O)-)), Entity1 = biotinyl, Entity2 = linker-carboxyfluorescein wherein linker = -NH(CH₂)₃O(CH₂)₂O-(CH₂)₂O(CH₂)₃NH-, x₁ = 1, x₂ = 8

The compound of Example 15 (9 mg) was dissolved in NMP (100 μ l), and DIPEA (20 μ l) and (13-carboxyfluoresceinylamido)-4,7,10-trioxotridecane-1-amine (13 mg) were added. After 10 minutes at 55°C, the crude product was 5 isolated by precipitation with ether. Yield: 5.3 mg (30%) after RP-HPLC purification. Mw. calculated for $C_{468}H_{682}N_{48}O_{157}S$ 9504.29. Found 9502.1 (MH^+ , MALDI-TOF MS).

EXAMPLE 17

10

Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(carboxymethyl)])], Figure 8, No. 17

Partly protected dendrimer of the form Entity1-A-(free acetic acid)₁₆, dendritic core A = 15 groups, each = $-NH(CH_2)_3O(CH_2)_2O(CH_2)_2O(CH_2)_3N(CH_2C(O))_2-$, Entity1 = Boc, 20 $x_1 = 1$, dendritic core terminates in free acid form

The compound of Example 8 (100 mg) was dissolved in 2 ml methanol and 30 mg Pd/C was added. The mixture was hydrogenated under atmospheric pressure for 1 hour. The 25 catalyst was filtered off, and the mixture reduced to dryness. Yield: 77 mg (100%). Mw. calculated for $C_{215}H_{384}N_{30}O_{85}S$ 4749.3. Found 4752.3 (MH^+ , MALDI-TOF MS).

EXAMPLE 18

30

Preparation of 1-(tert-butoxycarbonyl)-1,15-diaza-4,7,10-trioxapentadecane-15-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis(3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-17-bis[3,17-diaza-2-oxo-7,10,13-trioxaheptadecane-16-(2,6-dioxomorpholin-4-yl)])], Figure 8, No. 18

Activated dendrimer of the form Entity1-A-(activated group)₈, dendritic core A = 15 groups with 7 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, and the peripheral 8 = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-(2,6-dioxomorpholine, Entity1 = Boc, x1 = 1, dendrimer terminates in activated groups

The compound of Example 17 (10 mg) was dissolved in pyridine (100 μ l) and diisopropylcarbodiimide (4 μ l) was added. After 30 minutes at 55°C, the product was 10 precipitated by addition of ether. Yield: 9 mg (91%). Mw. calculated for C₂₁₅H₃₆₄N₃₀O₆₅ 4749.34. Found 4751.2 (MH⁺, MALDI-TOF MS).

EXAMPLE 19

15

Preparation of a dendrimer of the form (free amine)-A-(Entity2)₈, Figure 8, No.

Dendritic core A comprising 15 groups, 7 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, the peripheral 8 = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-(N(CH₂COOH)(CH₂C(O)-)), Entity2 = peptide nucleic acid having the nucleobase sequence carboxyfluoresceinyl-AAAACGGTCACCAGG-linker-Lys-NH₂- wherein linker = -NH(CH₂)₂O(CH₂)₂OCH₂C(O)-, x2 = 8, 25 Entity2 is coupled to A via the ϵ -N on Lys, dendritic core terminates in a free amine group

The peptide nucleic acid (carboxyfluoresceinyl-AAAACGGTCACCAGG-linker-Lys-NH₂) (2 mg) was dissolved in 30 NMP (100 μ l), TFA (20 μ l) and DIPEA (50 μ l). 0.2 mg of the compound of Example 18 was added and the mixture kept at 55°C for 30 minutes. The crude product was precipitated by addition of ether, then suspended in TFA (100 μ l) and re-precipitated. The product was purified by 35 ultracentrifugation, the excess unreacted peptide nucleic acid passing through a 30 kDa cut-off filter. Yield: 1 mg

(56%). Mw. calculated for $C_{1754}H_{2216}N_{798}O_{483}$ 42204.7. Found 42200 (MH^+ , MALDI-TOF MS).

EXAMPLE 20

5

Preparation of a dendrimer of the form (free amine)-A-(Entity2)₈, Figure 9, No. 20

Dendritic core A comprising 15 groups, 7 = $-NH(CH_2)_3O-(CH_2)_2O(CH_2)_2O(CH_2)_3N(CH_2C(O))_2-$, the peripheral 8 = $-NH(CH_2)_3O(CH_2)_2O(CH_2)_2O(CH_2)_3-(N(CH_2COOH)(CH_2C(O)-))$, Entity2 = peptide nucleic acid having the nucleobase sequence carboxyfluoresceinyl-TAACCTAACCTAACCC-linker-Lys-NH₂- wherein linker = $-NH(CH_2)_2O(CH_2)_2OCH_2C(O)-$, x2 = 8, Entity2 is coupled to A via the ε-N on Lys, dendritic core terminates in a free amine

The peptide nucleic acid (carboxyfluoresceinyl-TAACCTAACCTAACCC-linker-Lys-NH₂) (1 mg) was dissolved in NMP (100 μl), TFA (20 μl) and DIPEA (50 μl). 0.1 mg of the compound of Example 18 was added and the mixture kept at 55°C for 30 minutes. The crude product was precipitated by addition of ether, then suspended in TFA (100 μl) and re-precipitated. The product was purified by ultracentrifugation, the excess unreacted peptide nucleic acid passing through a 30 kDa cut-off filter. Yield: 0.6 mg (60%). Mw. calculated for $C_{1986}H_{2560}N_{854}O_{591}$ 47530.1. Found 47600 (MH^+ , MALDI-TOF MS).

30 EXAMPLE 21

Preparation of a dendrimer of the form (Entity1)-A-(Entity2)₈, Figure 9, No. 21

35 Dendritic core A comprising 15 groups, 7 = $-NH(CH_2)_3O-(CH_2)_2O(CH_2)_2O(CH_2)_3N(CH_2C(O))_2-$, the peripheral 8 =

-NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-(N(CH₂COOH)(CH₂C(O)-)), Entity1 = carboxyfluoresceinyl-TTTGCCAGT-Lys, Entity2 = peptide nucleic acid having the nucleobase sequence carboxyfluoresceinyl-TAACCTAACCTAACCC-linker-Lys-NH₂-
5 wherein linker = -NH(CH₂)₂O(CH₂)₂OCH₂C(O)-, and x1 = 1, x2 = 8, Entity1 is cross-linked to A via an EDTA, Entity2 is coupled to A via the ε-N on Lys

Excess EDTA dianhydride (1 mg) is reacted with
10 carboxyfluoresceinyl-TTTGCCAGT-Lys-NH₂ (1 mg) in NMP (100 μl), TFA (20 μl) and DIPEA (50 μl) to yield carboxyfluoresceinyl-TTTGCCAGT-Lys(ε-N-EDTA monoanhydride)-NH₂ which is isolated by ether precipitation. This activated intermediary compound is re-dissolved in
15 NMP (50 μl) and added to the dendrimer of Example 20 (0.6 mg) in 100 μl NMP, 20 μl TFA and 50 μl DIPEA at 55°C. After 1 hour, the product is precipitated with ether. The product was purified by ultracentrifugation, the excess activated intermediary compound passing through a 30 kDa
20 cut-off filter. Yield: 0.3 mg (46%). Mw. calculated for C₂₁₃₁H₂₇₃₀N₉₀₉O₇₁₆ 50966.9. Found 51100 (MH⁺, MALDI-TOF MS).

EXAMPLE 22

25 Preparation of a dendrimer of the form (free amine)-A-(Entity2)₈, Figure 10, No. 22

Dendritic core A comprising 15 groups, 7 = -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-, the peripheral 8 =
30 -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃-(N(CH₂COOH)(CH₂C(O)-)), Entity2 = linker-carboxyfluorescein wherein linker = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃NH-, x2 = 8

The compound of Example 18 (10 mg) was dissolved in NMP (100 μl), and DIPEA (20 μl) and (13-carboxyfluoresceinylamido)-4,7,10-trioxotridecane-1-amine (13 mg) were

added. After 10 minutes at 55°C, the crude product was isolated by precipitation with ether and subsequently dissolved in 100 µl TFA and re-precipitated with ether. Yield: 7 mg (36%) after RP-HPLC purification. Mw. 5 calculated for C₄₅₈H₆₄₈N₄₆O₁₅₅ 9278.4. Found 9281.5 (MH⁺, MALDI-TOF MS).

EXAMPLE 23

10 Preparation of dendrimer of the formula (free amine)-A-
(Entity2)₈, Figure 11, No. 23

Dendrimer wherein the dendritic core A comprises 15 groups, 7 = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-,-, 15 the peripheral 8 = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃- (N(CH₂COOH)(CH₂C(O)-)), Entity2 = AAL-AAL-linker- 20 carboxyfluorescein wherein linker = -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃- (N(CH₂COOH)(CH₂C(O)-)), x2 = 8, dendrimer terminates in a free amine

(13-Carboxyfluoresceinylamido)-4,7,10-trioxotridecane-1-amine, 57 mg, was dissolved in 0.5 mL NMP and 0.1 mL DIPEA. The compound of Example 31, 50 mg in 250 µL 25 pyridine, was added. After 5 min the intermediate product was precipitated with ether, deprotected with 0.5 mL TFA, precipitated, neutralised with 0.1 mL DIPEA and dissolved in 0.5 mL NMP. It was again reacted with 75 mg of compound 31 in 375 µL pyridine for 5 min, followed by 30 precipitation and 5 min TFA deprotection. Finally HPLC purification afforded 25 mg of AAL-AAL-linker-carboxy-fluorescein. MALDI-TOF MS: Found 1214. Calculated 1214.1 (M⁺H). This was reacted with the compound of Example 18 (10 mg) in NMP (100 µL) and DIPEA (30µL) for 10 min at 35 room temperature. Precipitation with ether followed by final TFA deprotection and HPLC purification gave 15 mg

of the title compound. MALDI-TOF MS: Found 14460. Calculated 14445.

EXAMPLE 24

5

Preparation of N-Boc,N' (bromoacetyl)-4,7,10-trioxotri-decanediamine, Figure 12, No. 24

The compound of Example 1, 16 g, 50 mmol, was dissolved in 50 mL DCM and added dropwise over 30 min to 55 mmol bromoacetic anhydride and 70 mmol lutidin in 200 mL ice cold DCM. The mixture was extracted twice with 100 mL 1 M citrate pH 4.5, reduced to partial dryness, and immediately purified with 5% MeOH in DCM. Yield 17.6 g, 80%. MALDI-TOF MS (M⁺H-Boc): Found 342.2. Calculated 343.

15

EXAMPLE 25

Preparation of DiAminoAcidLinker-dibenzyl ester (DAAL-dibenzyl ester)

20

Dendrimer of the formula (Entity1)₂-A-(Entity2)₂, wherein Entity1 = Boc, Entity2 = OBz, x₁ = 1, x₂ = 2, and A = DAAL

25

The compound of Example 4, 5.15 g free amine form, 10 mmol, was dissolved in 30 mL DMF and 15 mL DIPEA. N-Boc,N' (bromoacetyl)-4,7,10-trioxotridecanediamine, 10 g, was added and the mixture stirred for 2 h at 50 C. The solvent was removed under reduced pressure, the mixture taken up in 100 mL DCM and extracted with 100 mL sodium hydrogen carbonate, then 100 mL water. Reduction to dryness and subsequent chromatography with 5-15% MeOH in DCM gave 6.8 g, 55%, as a viscous oil. MALDI-TOF MS (M⁺H): Found 1237.3. Calculated 1238.2.

35

EXAMPLE 26

Preparation of DiAminoAcidLinker-dibenzyl ester (DAAL-dibenzyl ester), free amine form, Figure 12, No. 26

5 Dendrimer of the formula (free amine)₂-A-(Entity2)₂,
wherein Entity2 = OBz, x2 = 2, and A = DAAL

DiAminoAcidLinker-dibenzyl ester, 6.8 g, was dissolved in 30 mL TFA. After 5 min TFA was removed under reduced pressure, and the mixture taken up in 200 mL DCM and extracted twice with 150 mL sodium carbonate. The organic phase was reduced to dryness and further dried by evaporation from dry toluene under reduced pressure. Yield 4.8 g, 84%. MALDI-TOF MS: Found 1038.6. Calculated 15 1038.1.

EXAMPLE 27

Preparation of TetraAminoAcidLinker-dibenzyl ester (TAAL-dibenzyl ester), Figure 13, No. 27

Dendrimer of formula (Entity1)₄-A-(Entity2)₂, wherein Entity1 = Boc, Entity2 = OBz, A = 3 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂-, and 2 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O)-)₂

The compound of Example 26, 4.8 g, was dissolved in 20 mL DMF and 10 ML DIPEA. 10 g of N-Boc,N'(bromoacetyl)-4,7,10-trioxotridecanediamine was added and the mixture 30 stirred for 4 h at 50°C. Removal of the solvent under reduced pressure gave an oil that was taken up in 100 mL DCM and extracted with sodiumhydrogencarbonate, followed water. The organic phase was removed, and the residue chromatographed on silica with first 15% MeOH in 35 ethylacetate, and subsequently eluted with 15% MeOH in DCM. The so obtained product was purified on silica with

10% ammonia saturated MeOH in DCM to give 2.2 g, 19%, of a viscous clear oil. MALDI-TOF MS: Found 2480. Calculated 2479.

5 EXAMPLE 28

Preparation of TetraAminoAcidLinker (TAAL), free diacid form, Figure 13, No. 28

10 Dendrimer of the form (Entity1)₄-A-(free acetic acid)₂, wherein Entity1 = Boc, x1 = 4, A = TAAL

15 The TetraAminoAcidLinker-dibenzyl ester of Example 27, 1.2 g, was dissolved in 20 mL MeOH, 200 mg 10%Pd/C was added, and the mixture hydrogenated for 2 h. Catalyst and solvent were removed by centrifugation and subsequent evaporation to give 1 g of the product. MALDI-TOF MS: Found 2300. Calculated 2299.

20 EXAMPLE 29

Preparation of TetraAminoAcidLinker-anhydride, Figure 13, No. 29

25 Dendrimer of the structure (Entity1)₄-A-(N-2,6-dioxomorpholine), wherein Entity1 = Boc, x1 = 4, A = 3-NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂-, dendrimer terminates a N-2,6-dioxomorpholine group

30 TetraAminoAcidLinker, free diacid form, of Example 27, 100 mg was dissolved in 1 mL pyridine. 0.1 mL diisopropylcarbodiimide was added and the mixture reacted at 70°C for 10 min. This approx 0.38 M solution of the product was used immediately without further purification.

35 Reaction of such solution with excess free primary amine

under basic conditions indicated >90% conversion to anhydride.

EXAMPLE 30

5

Preparation of TetraAminoAcidLinker-dibenzylester-free tetraamine

Dendrimer of the structure (free amine)4-A-(Entity2)2, wherein Entity2 = OBz, X2 = 2, A = 3 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂-, and 2 -NH(CH₂)₃O-(CH₂)₂O-(CH₂)₂O(CH₂)₃N(CH₂C(O)-)₂, dendritic core terminates in a free amine

15 TetraAminoAcidLinker-dibenzyl ester, 230 mg, 0.1 mmol, was dissolved in 1 mL TFA. After 5 min, 7 mL ether was added, and the product isolated by centrifugation. It was repeatedly vortexed with ether to afford a 300 mg of a semisolid TFA-adduct, that was used immediately without further purification. MALDI-TOF MS: Found 2078.

20 Calculated 2079.

EXAMPLE 31

25 Preparation of AminoAcidLinker-anhydride (AAL-anhydride)

The compound of Example 3, 53 mg in 0.25 mL pyridine, was activated with 25 μ L diisopropylcarbodiimide for 10 min at 70°C, and used in solution without further purification. The yield was assumed to be quantitative,

30 50 mg.

EXAMPLE 32

35 Preparation of O-(Boc-aminopropyl)-O'-aminopropyl-ethyl- eneglycol (Δ 34 glycol units)

10 O,O'-Bi¹⁴aminopropyl-ethyleneglycol was dissolved in 250 mL THF. The mixture was cooled on an ice bath and 1,18 g Boc-anhydride in 15 mL THF was added drop-wise, and the mixture reduced to 20 g of a viscous oil. This oil was 5 purified on silica with 20% MeOH in DCM, then eluted with 20% half ammonia saturated MeOH in DCM. The product was re-purified with 15 % half ammonia saturated MeOH in DCM to give 2.75 g, 29 %. MALDI-TOF MS: Found (44.05)_n + 235. Calculated (44.05)_n + 233.

10

EXAMPLE 33

Preparation of O-(Boc-aminopropyl)-O'-(bromoacetamidopropyl)-ethyleneglycol (Δ 34 glycol units)

15

15 O-(Boc-aminopropyl)-O'aminopropyl-ethyleneglycol (Δ 34 glycol units), 1.1 g, was dissolved in 3 mL DCM and added to 0.7 mmol bromoacetic anhydride and 1 mmol lutidine in 3 mL ice cold DCM. After 15 min reaction the mixture was 20 extracted twice with 2 mL 1 M sodium citrate, pH 4.5, and following reduction to dryness, it was purified on silica with 15% MeOH in DCM. Yield 1 g, 85%. MALDI-TOF MS (M-Boc): Found (44.05)_n + 252. Calculated (44.05)_n + 253.

25 EXAMPLE 34

Preparation of Octa-(Boc-amino-PEG)-dendrimer-dibenzyl-ester

30 Protected dendrimer of form (Entity1)₈-A-(Entity2)₂, wherein Entity1 = Boc, Entity2 = OBz, x1 = 8, x2 = 2, A = 3 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂-, and 2 - NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O)-)₂, and 8 - NH(CH₂)₃O(CH₂CH₂O)₃₄(CH₃)NH-

35

TetraAminoAcidLinker-dibenzylester-free tetraamine TFA adduct, 100 mg, approximately 0.03 mmol, was dissolved in 1 mL DMF and 0.5 mL DIPEA. O-(Boc-aminopropyl)-O'-(brom-acetamidopropyl)-ethyleneglycol (Δ 34 glycol units) 1g, 5 approximately 0.5 mmol, was added and the mixture stirred for 4 h at 60°C. 5 mL DCM was added and the mixture extracted with 2 mL sodium hydrogen carbonate, then with 1 mL water. DCM was evaporated off, and the product precipitated by addition of 12 mL ether. Yield 400 mg. 10 MALDI-TOF MS: Found: 16600 (8 PEG-arms) and as a major impurity 12900 (6 PEG-arms).

EXAMPLE 35

15 Preparation of Octa-(carboxyfluoresceinamido-PEG)-dendrimer-dibenzylester, Figure 15, No. 35

Dendrimer of the form (Entity1)_{x1}-A-Entity2, wherein Entity1 = Fluorescanyl, Entity2 = OBz, x1 = 8, x2 = 1, A = 3 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂-, and 2 NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O)-)₂, and 8 NH(CH₂)₃O(CH₂CH₂O)₃₄(CH₃)NH-

25 Octa-(Boc-amino-PEG)-dendrimer-dibenzylester, 200 mg, was dissolved in 1 mL TFA and after 5 min the intermediate free octa-amine was precipitated by addition of 7 mL ether. It was vortexed with ether, then dissolved in 1 mL NMP and 0.3 mL DIPEA. N-hydroxy-succinimido-carboxy-fluorescein, 50 mg, was added, and after 10 min 30 additional 50 mg was added. After 30 min in all, the product was precipitated by addition of 7 mL ether. MALDI-TOF MS: Found: 18600 (major product, consistent with 8 fluorescinylated PEG arms).

35 EXAMPLE 36

Preparation of Octa-(carboxyfluoresceinamido-PEG)-dendrimer-free diacid, Figure 15, No. 36

Dendrimer of the form (Entity1)₈-A-(free acid)₂, Entity1
5 = Fluorescanyl, x1 = 8, A = 3 -NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂-, and 2 NH(CH₂)₃O-(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O)-)₂, and 8
NH(CH₂)₃O(CH₂CH₂O)₃₄(CH₃)NH-

10 Octa-(carboxyfluoresceinamido-PEG)-dendrimer-dibenzyl ester of Example 35, approximately 100 mg, was dissolved in 1 mL 1.0 M LiOH and 2 mL THF. After 30 min reaction, 1 mL 1.0 M HCl was added. Further 6 mL of water was added, and addition of ammonia to pH 8 afforded an almost black
15 (intensely red) but clear solution. RP-HPLC allowed isolation of the title compound having 8 fluorescein groups in the outermost layer (eluting with approximately 55% acetonitrile in 0.1% aqueous TFA) in 18 mg yield. MALDI-TOF MS: Found 18400. Further 30 mg of dendrimers
20 with 6 and 7 fluoresceinylated arms were also isolated.

EXAMPLE 37

Preparation of Octa-(carboxyfluoresceinamido-PEG)-dendrimer-anhydride, Figure 15, No. 37

Dendrimer of the form (Entity1)₈-A-(N-2,6-dioxomorpholine)₂, Entity 1 = fluorescanyl, x1 = 8, A = 1 N(CH₃)₂O(CH₂)₂O(CH₂)₂O(CH₃)₃,
30 3 N(CH₃)₂O(CH₂)₂O(CH₂)₂O(CH₃)₃NHC(O)CH₂, 8 NH(CH₂)₃O(CH₂CH₂O)₃₄(CH₂)₃NHC(O)CH₂, dendrimer terminates in dioxomorpholine groups

Octa-(carboxyfluoresceinamido-PEG)-dendrimer-free diacid, 35 3 mg in 50 μL NMP, was reacted with 5 μL diisopropylcarbodiimide for 15 min at room temperature and precipitated

by addition of 1 mL ether. It was used without further purification. A control reaction with a large excess of the compound of Example 32 (O-(Boc-aminopropyl)-O'aminopropyl-ethyleneglycol (Δ 34 glycol units)) indicated that 5 approximately 85% of the product was activated this way.

EXAMPLE 38

Preparation of Boc-amino PEG

10

50 g of PEG 1000, 50 mmol, was dissolved in 300 mL THF and 10 mL 2M, butylllithium was added. The compound of Example 24, 9 g in 30 mL THF, was added and the solution stirred overnight under nitrogen. Reduction to dryness, 15 extraction with hydrogen carbonate from DCM and repeated chromatography with methanol in DCM afforded 12g 50 %. MALDI-TOF MS: Found (44.053)_n

EXAMPLE 39

20

Preparation of 1-(bromoacetyl)-1,15-diaza-4,7-10-trioxa-pentadecane-15-bis(benzyloxycarbonylmethyl)

25

Dendrimer of the form (Entity 1)_{x1}-A-(Entity 2)_{x2}, Entity 1 = bromoacetyl, x1 = 1, Entity 2 O-Bz, x2 = 2
A = 1 NH(CH₃)₂O(CH₂)₂O(CH₂)₂O(CH₃)₃N((CH₂)C(O))₂

30

The TFA adduct of Example 4 was extracted with hydrogen carbonate from DCM and dried by evaporation from toluene under reduced pressure, to give the free amine in 80 % yield. 10.3 g, 20 mmol of this product was dissolved in 25 mL toluene and added drop-wise over 30 min to 22 mmol bromoacetic anhydride and 30 mmol lutidin in 100 mL ice cold DCM. The mixture was extracted twice with 100 mL 1 M citrate pH 4.5, reduced to partial dryness, and 35

immediately purified with 5% MeOH in DCM (r.f 0.8). Yield 80%. MALDI-TOF MS: Found 638.7. Calculated 638.

EXAMPLE 40

5

Preparation of Boc-PEG-linker dibenzylester

Dendrimer of the form (Entity1)_{x1}-A-(Entity2)_{x2}, wherein Entity1 = Boc, Entity2 = OBz, x1 = 1, x2 = 2, A = -
10 NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂(O(CH₂)₂)₂₂OCH₂C(O)-
NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂

Boc-amino-PEG of Example 38, 12 g, was dissolved in 60 mL DCM and 5.5 mL 2 M butyllithium was added. With cooling 15 4.4 g of 1-(bromoacetyl)1,15-diaza-4,7-10-trioxapentadecane-15-bis(benzyloxycarbonylmethyl) in 10 mL THF was added and the mixture stirred overnight at room temperature. Reduction to dryness, extraction with hydrogen carbonate and repeated chromatographic work-up 20 afforded 1.7 g 10%. MALDI-TOF MS:

EXAMPLE 41

Preparation of Boc-PEG-linker free diacid
25 Dendrimer of the form (Entity1)_{x1}-A wherein Entity1 = Boc, x1 = 1, A = NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂(O(CH₂)₂)₂₂OCH₂C(O)-
NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂ Dendrimer terminates
30 in free acids

Boc-PEG-linker dibenzylester of Example 40, 1.7 g, 1 mmol, was dissolved in 15 mL MeOH. Under nitrogen 300 mg 10% Pd/C was added and the mixture hydrogenated for 2 h. 35 Removal of catalyst and solvent gave a quantitative yield

of a viscous oil. This compound was stored as a 20% solution in pyridine, for subsequent reaction.

EXAMPLE 42

5

Preparation of Boc-Peg-linker anhydride

Boc-PEG-linker free diacid, 20% in pyridine, was activated with 10% diisopropylcarbodiimide for 10 min at 10 70°C to afford an approx. 1M solution of the anhydride which was used fresh.

EXAMPLE 43

15 Preparation of N-Boc-N'-bromoacetyl-ethylenediamine

16 g mono-Boc-ethylenediamine, 100 mmol, was dissolved in 50 mL DCM and added drop-wise over 30 min to 110 mmol bromoacetic anhydride and 130 mmol lutidin in 350 mL ice 20 cold DCM. The mixture was filtered and extracted twice with 200 mL 1 M citrate pH 4.5, filtered, reduced to partial dryness and stirred on an ice bath with 150 mL ether. The product was filtered off and dried. Yield 10 g, light tan powder.

25

EXAMPLE 44

Preparation of N-Boc-N'-(N-piperazinyl-acetyl)-ethylenediamine

30

4.3 g piperazine was dissolved in 50 mL THF and 2.8 g N-Boc-N'-bromoacetyl-ethylenediamine was added. After 1 h the mixture was reduced to dryness and taken up in 50 mL DCM and extracted once with water. Reduction to dryness 35 and chromatographic workup with 10% ammonia saturated

MeOH in DCM gave 1.4 g, 50% as an oil that slowly solidified.

EXAMPLE 45

5

Preparation of Boc-piperazine-linker benzylester

N-Boc-N'-(N-piperazinyl-acetyl)-ethylenediamine, 1.4 g, was dissolved in 5 mL DMF and 1 mL DIPEA, 1.5 mL was 10 added, followed by 1.2 g bromoacetic acid benzylester. The mixture was stirred for 30 min at 40°C, taken up in 50 mL DCM and extracted with 50 mL sodium hydrogen carbonate. Chromatography with 5% MeOH in DCM afforded 800 mg, 36%. MALDI-TOF MS (M⁺H): Found 438.7. Calculated 15 439.4.

EXAMPLE 46

Preparation of Boc-piperazine-linker, free acid
20 Boc-piperazine-linker benzylester, 800 mg, was dissolved in 10 mL MeOH. Under nitrogen 100 mg 10%Pd/C was added and the mixture hydrogenated for 1 h. Pd/C was removed by centrifugation, and subsequent evaporation of the solvent 25 afforded the title compound in quantitative yield, 620 mg. MALDI-TOF MS (M⁺H): Found 347.1. Calculated 347.4.

EXAMPLE 47

30 Preparation of PEG34-linker-dibenzylester
Dendrimer of the form (Entity1)_{x1}-A-(Entity2)_{x2}, wherein Entity1 = Boc, Entity2 = OBz, x1 = 1, x2 = 2, A = - NH(CH₂)₃(O(CH₂)₂)₃₄O(CH₂)₃N(CH₂C(O))₂
35 O-(Boc-aminopropyl)-O'aminopropyl-ethyleneglycol (Δ 34 glycol units), 1.49 g, was dissolved in 5 ml DMF and 1.5

mL DIPEA. 616 μ L bromoacetic acid benzylester was added and the mixture reacted for 1 h at 60°C. The solvent was removed, and the residue taken up in 25 mL DCM. It was extracted with 25 mL sodium hydrogen carbonate, which was 5 in turn back-extracted with 25 mL DCM. The combined organic phases were reduced and purified on silica with 10% MeOH in DCM, eluding with 15% MeOH in DCM. Yield 810 mg, 46% as a clear viscous oil. MALDI-TOF MS (M-Boc): Found $(44.05)_n + 429$. Calculated $(44.05) + 430$.

10

EXAMPLE 48

Preparation of PEG34-linker-free diacid

15 Dendrimer of the form $(\text{Entity1})_{x1}-\text{A}$, wherein Entity1 = Boc, $x1 = 1$, $\text{A} = -\text{NH}(\text{CH}_2)_3(\text{O}(\text{CH}_2)_2)_{34}\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{C}(\text{O}))_2$ dendrimer terminates in free acids.

20 PEG34-linker-dibenzylester, 0.81 g, was dissolved in 20 mL MeOH and 160 mg Pd/C was added. The mixture was hydrogenated for 2 h, and following removal of Pd/C and solvent, 723 mg of the free diacid was obtained (98%).

25

EXAMPLE 49

Preparation of 49 PEG34-linker-anhydride

30 95.6 μ l of a 20% solution of PEG34-linker-free diacid in pyridine was reacted with 9.65 μ L diisopropylidimine for 10 min at 70°C and used without further purification.

EXAMPLE 50

35 Preparation of dendrimer of the formula $((\text{FluPEG})_4\text{TAAL})_4\text{TAAL-PEG-PEG-PNA}$, wherein Entity1 = FluPEG, $x1 = 16$, Entity 2 = PNA, wherein the peptide

nucleic acid has the nucleobase sequence TGT GCG CCC TCA
ACT AAC, x2 = 1

5 This procedure was also used for preparing the compounds
of Examples 50-57.

The piperazine linker of Example 46 was directly coupled
to the PNAs using standard solid phase Boc chemistries.

10 The other linkers were coupled in solution to the PNAs
and derivatives using the following general procedure:
The PNA was dissolved in NMP to a concentration of 1-5
mM. If this could not be achieved in neat NMP, addition
of 20% water resulted in a clear solution of desired
15 concentration. Residual TFA from the HPLC purification of
the PNAs was then neutralised by addition of 5% pyridine.
2 equivalents of the appropriate Boc-protected linker-
anhydride was added, followed by addition of 20% DIPEA.
The reaction was followed by MALDI, in case of incomplete
20 reaction after 10 min (observed mainly when using water
as co-solvent due to competing hydrolysis), additional 2
equivalents of linker were added.

25 The crude product was precipitated by addition of 10x
excess of ether.

30 Boc-deprotection was achieved by dissolving in 50-100x
excess 5% meta-cresol in TFA for 10 min, followed by
precipitation with further 10x excess ether.

35 Either additional linkers were attached to the PNA-
constructs, if necessary following HPLC purification, or
the constructs were labelled using commercially available
amino-reactive labels in highest possible concentration,
typically 3-15 weight % label, typically 3-30x molar
excess of label relative to free amino groups.

EXAMPLE 51

5 Preparation of dendrimer of the formula ((FluPEG)₄TAAL-PEG)₄TAAL-PEG-PNA, wherein Entity1 = FluPEG, x1 = 16, Entity 2 = PNA, wherein the peptide nucleic acid has the nucleobase sequence TGT GCG CCC TCA ACT AAC, and x2 = 1

10 The product was prepared according to the procedure described in Example 50.

EXAMPLE 52

15 Preparation of a dendrimer of formula (FluPEG)₄TAAL-PNA-Lys-Cy3, wherein Entity1 = FluPEG, x1 =4, Entity2 = Cy3, x2 = 1, Lys = lysine, and wherein PNA is a peptide nucleic acid with the nucleobase sequence TGT GCG CCC TCA ACT AAC

20 The product was prepared according to the procedure described in Example 50.

EXAMPLE 53

25 Preparation of a dendrimer complex of formula (label-PEG)₄TAAL-PNA-Lys-TAAL(PEG-label)₄, wherein Entity1 = label-PEG, the label being Flu (fluorescein), Rho (rhodamine), Lis (lissamine), Cy3, or DNP, x1 = 4, Dendritic core A = TAAL coupled to PNA-Lys coupled to
30 another dendritic core A = TAAL, Entity1 = PEG-label, x1 = 4, and wherein the peptide nucleic acid, PNA, has the nucleobase sequence TGT GCG CCC TCA ACT AAC

35 The product was prepared according to the procedure described in Example 50.

- A: Label being Flu
- B: Label being Rho
- C: Label being Lis
- D: Label being Cy3
- 5 E: Label being DNP

EXAMPLE 54

Preparation of dendrimer complex of formula
10 ((FluPEG)₄TAAL)₄TAAL-PNALys-TAAL(TAAL)₄(PEGFlu)₄, wherein
Entity1 = FluPEG, x1 = 16, dendritic core coupled to PNA-
Lys coupled to another dendritic core, wherein Entity1 =
FluPEG, x1 = 16, and wherein the peptide nucleic acid PNA
has the nucleic acid sequence A: CCA TAT GCA GTT ATA AGT
15 AGG, B: TAT TGT ACC AAG CAG AGT ACC, C: GGT ATA TAT AAG
ATG ACA CAG GA, and D: GTT AGT TAT ATT GGG TGA TAT GT

The product was prepared according to the procedure
described in Example 50.

20

EXAMPLE 55

Preparation of dendrimer complex of the formula
25 (Flu(AAL)₂)₄TAAL-PNALys-TAAL((ALL)₂Flu)₄, wherein Entity1 =
Flu(AAL)₂, x1 = 4, dendritic core coupled to PNA-Lys
coupled to another dendritic core, wherein Entity1 =
Flu(AAL)₂, x1 = 4, wherein the peptide nucleic acid PNA
has the nucleic acid sequence A: CCA TAT GCA GTT ATA AGT
AGG, B: TAT TGT ACC AAG CAG AGT ACC, C: GGT ATA TAT AAG
30 ATG ACA CAG GA, and D: GTT AGT TAT ATT GGG TGA TAT GT

The product was prepared according to the procedure
described in Example 50.

35 EXAMPLE 56

Preparation of dendrimer complex of the formula (Label-PEG)₄TAAL-PNALys-TAAL(PEG-label)₄, wherein Entity1 = labelPEG, x1 = 4, dendritic core coupled to PNA-Lys coupled to another dendritic core, wherein Entity1 = labelPEG, x1 = 4, and wherein the peptide nucleic acid PNA has the nucleic acid sequence A: CCA TAT GCA GTT ATA AGT AGG, B: TAT TGT ACC AAG CAG AGT ACC, C: GGT ATA TAT AAG ATG ACA CAG GA, and D: GTT AGT TAT ATT GGG TGA TAT GT

10 The product was prepared according to the procedure described in Example 50.

EXAMPLE 57

15 Preparation of a dendrimer complex of the formula (FluPEG)₄TAAL-PIP-PIP-PNA-PIP-PIP-Lys-TAAL(FluPEG)₄, wherein Entity1 = FluPEG, x1 = 4, dendritic core A coupled to PIP-PIP-PNA-PIP-PIP-Lys coupled to a dendritic core A, wherein Entity1 = FluPEG, x1 = 4, and wherein the peptide nucleic acid, PNA, has the nucleobase sequence TGT GCG CCC TCA ACT AAC

20 The product was prepared according to the procedure described in Example 50.

25

EXAMPLE 58

30 Preparation of a dendrimer complex of formula (Flu-PEG)₄TAAL-PNA-Lys-TAAL(PEG-Flu)₄, wherein Entity1 = label-PEG, x1 = 4, Dendritic core A = TAAL coupled to PNA-Lys coupled to another dendritic core A = TAAL, Entity1 = PEG-label, x1 = 4, , and wherein the peptide nucleic acid, PNA, has the nucleobase sequence A: TuGTu GCG CCC TuCD DCT DDC

35

The product was prepared according to the procedure described in Example 50.

EXAMPLE 59

5

Preparation of a dendrimer complex of formula (Rho-PEG)₄TAAL-PNA-Lys-TAAL(PEG-Rho)₄, wherein Entity1 = label-PEG, x1 = 4, Dendritic core A = TAAL coupled to PNA-Lys coupled to another dendritic core A = TAAL, Entity1 = PEG-label, x1 = 4, , and wherein the peptide nucleic acid, PNA, has the nucleobase sequence GTuTu DGTu TuGD GGG CGC DCD

10 The product was prepared according to the procedure
15 described in Example 50.

EXAMPLE 60

Evaluation of the probes

20

The experiment was carried out on metaphase chromosome spreads using the dendrimer complexes of examples 50-59 and single-labelled conventional peptide nucleic acid probes having a corresponding nucleobase sequence. The 25 samples were denatured in 60% formamide at 80°C for 10 minutes. Then the dendrimer complexes or the single-labelled probes in hybridisation buffer (as defined in Example 65) was added, and the slides were allowed to cool to room temperature, following stringent wash at 30 55°C. The probes were scored on a scale from 1 to 5, the signal obtained from the single-labelled set to 2.

Score	Dendrimer or dendrimer complex
2	Single-labelled probe (2, 6)
3	Example 50
2	Example 51
3	Example 52
3	Example 53 A
3	Example 53 B
3	Example 53 C
4	Example 53 D
4	Example 57
3	Example 58
3	Example 59
5	Mix of probes from Example 58 + 59
2	Single-labelled probes (mix of 4)
1	Example 54 A-D, mix of 4
2	Example 55 A-D, mix of 4
3	Example 56 A-D, mix of 4

EXAMPLE 61

5

Preparation of dendritic complexes of the form VSDEX:(CD_x)_n:(dendrimer of example 22)_m; where VSDEX is a linear vinylsulfone activated dextran of M.W. 150.000 and CD_x are antibodies; CD3, CD4, CD 19, CD23, CD33, CD34
10 class I, CD56 all DAKO A/S.

General procedure:

96 nmol of dendrimer of Example 22 and 3 nmol VSDEX were reacted in 0.28 mL 0.14 M Sodium hydrogen carbonate
15 buffer for 6 h. at 30 C. 15 nmol antibody was then added, and the reaction was allowed to proceed for additional 18 h. Unreacted vinylsulfones were quenched by amino containing stop buffer for 1 h. The conjugates were then

purified on a sepharyl S-200HR column. Relative absorbancies at 278 and 498 nm were used to determine the stoichiometries of the conjugates. Typical conjugation ratios obtained were 2-3 antibodies/DEX and 3-4 octa-5 fluorescein dendrimers/DEX giving an antibody:fluorescein ratio around 1:10.

EXAMPLE 62

10 Preparation of dendritic complexes of the form VSDEX:(CD_x)_n:(dendrimer of example 23)_m; where VSDEX is a linear vinylsulfone activated dextran of M.W. 150.000 and CD_x are antibodies; CD3,CD4, CD 19, CD23, CD33, CD34 class I, CD56 all DAKO A/S.

15

General procedure:

96 nmol of dendrimer of Example 22 and 3 nmol VSDEX were reacted in 0.28 mL 0.14 M Sodium hydrogen carbonate buffer for 6 h. at 30 C. 15 nmol antibody was then added, 20 and the reaction was allowed to proceed for additional 18 h. Unreacted vinylsulfones were quenched by amino containing stop buffer for 1 h. The conjugates were then purified on a sepharyl S-200HR column. Relative absorbancies at 278 and 498 nm were used to determine the 25 stoichiometries of the conjugates. Typical conjugation ratios obtained were 2-3 antibodies/DEX and 1-2 octa-fluorescein dendrimers/DEX giving an antibody:fluorescein ratio around 1:5.

30 EXAMPLE 63

Comparison of VSDEX:(CD3)₃:(dendrimer of example 22)₄ v.s. VSDEX:(CD3)₃:(dendrimer of example 23)₂ v.s CD23:FITC (DAKO A/S)

35

The 3 conjugates were tested for staining of lymphocytes by flow cytometry. VSDEX:(CD3)₃:(dendrimer of example 22)₄ gave a significantly stronger specific signal than CD23:FITC, however there was also strong unspecific 5 staining of granulocytes.

VSDEX:(CD3)₃:(dendrimer of Example 23)₂ produced the strongest specific signal of the three conjugates, while unspecific staining of granulocytes was significantly 10 reduced relative to the :(CD3)₃:(dendrimer of example 22)₄.Indeed this conjugate could be diluted up to 16 times, while still producing a specific signal comparable to the plain CD23:FITC conjugate.

15 The fact that :(CD3)₃:(dendrimer of example 23)₂ gave a stronger signal than the VSDEX:(CD3)₃:(dendrimer of Example 22) ,despite a two fold higher fluorescein to antibody ratio is ascribed to the increased spacing of the fluoresceins in the dendrimer of Example 23. This 20 dendrimer features 137 bonds between closest fluoresceins, whereas the fluoresceins in the dendrimer of Example 22 are spaced by 67 bonds.

EXAMPLE 64

25

Preparation of Streptavidine:(dendrimer of example 37) conjugate

Dendrimer of example 37, 2 mg was dissolved in water and 30 added to 2 mg streptavidine in 0.1 mL 0.14M aqueous carbonate buffer, pH 10. After 10 min reaction the mixture was neutralized with citrate buffer to pH 7. The conjugate was purified on a sepharyl S-200HR colum, eluding after unconjugated streptavidine, but before 35 unconjugated dendrimer.

MALDI of the assumed conjugate showed a new peak at M.W. 31500, corresponding to a streptavidine subunit with one attached dendrimer. A peak at 13000 corresponding to unconjugated subunit was however also present, indicating 5 that under the reaction conditions, not all subunits were labeled.

EXAMPLE 65

10 Detection of γ -globin mRNA using the dendrimer of formula
of Example 19, and use as signal amplification system or
signal enhancement system

Human cord blood was isolated and formaldehyde fixed 15 immediately after delivery and immobilised on glass microscope slides. The slides were covered with a hybridisation solution (50 mM Tris, 10 mM NaCl, 10% w/v dextran sulphate, 30% w/v formamide, 0.1% Triton X-100[®], 5 mM EDTA, pH 7.6). The hybridisation solution further 20 comprised either the dendrimer of Example 19 (5 nM) or a peptide nucleic acid of the corresponding nucleobase sequence (40 nM), however being labelled with one fluorescein label (not a dendrimer). After 30 minutes hybridisation at 55°C, the slides were subjected to 25 stringent washing conditions using a washing buffer containing 30% w/v formamide, 0.1% Triton X-100[®], 5 mM EDTA for 30 minutes. The fluorescent signal was amplified using anti-FITC:HRP; biotinyl-tyramide; FITC-streptavidin. Following a final stringent wash, the 30 slides were dried and mounted with Imagen Mounting Fluid (DAKO A/S). The signal obtained with the dendrimer of Example 19 was approximately 10 times stronger than the signal obtained with the single-labelled peptide nucleic acid probe. Apparently, only one peptide nucleic acid of 35 the dendrimer binds to it's complementary mRNA sequence in the sample, the remaining 7 fluorescein-labelled

peptide nucleic acids contributing to the signal amplification. It should further be noticed that the nominal concentration of peptide nucleic acid in the dendrimer-containing hybridisation buffer and control 5 buffer containing single-labelled peptide nucleic acid is the same. Control experiments with adult male peripheral blood showed no increased background or cross-reaction of the dendrimer relative to the single-labelled peptide nucleic acid probe.

10

EXAMPLE 66

Use of the dendrimers in multi-layer detection systems

15 The dendrimers of Example 19 and Example 21 were used. The dendrimer of Example 19 was hybridised to human cord blood as described in Example 51. Entity1 on the dendrimer of Example 21 is complementary to the 8 Entity2's on the dendrimer of Example 19. This was 20 exploited in an extra signal-enhancing step. Following hybridisation with dendrimer of Example 19 and stringent washing, the dendrimer of Example 21 in hybridisation buffer was applied at room temperature followed by a stringent wash at 35°C. For comparison, this extra step 25 was omitted in case of one of the slides. An amplification of about 5 times was seen when comparing the signal in case of hybridisation with the dendrimers of both Example 19 and Example 21 with the signal in case of hybridisation only with the dendrimer of Example 19. 30 This corresponds to approximately 50 times enhancement compared to a single-labelled conventional probe (cf. Example 65).

EXAMPLE 67

35

Reduction of background signal

The experiment was carried out on metaphase chromosome spreads using the dendrimer of Example 20 and a single-labelled conventional peptide nucleic acid probe having a corresponding nucleobase sequence. The nucleobase sequence was directed at the human telomere sequence. The nucleobase sequence (TAACCC)₃. The samples were denatured in 60% formamide at 80°C for 10 minutes. Then the dendrimer or the probe in hybridisation buffer (as defined in Example 65) was added, and the slides were allowed to cool to room temperature. Following stringent wash at 60°C, almost identical signals were observed for the dendrimer and the conventional probe, respectively, because the target is saturated even with the conventional probe. Background signal was seen in both cases. Following stringent wash at 70°C, the signal was completely absent in the case of the conventional probe, whereas the dendrimer signal was undiminished. Furthermore, the background signal in case of the dendrimer was significantly reduced by the increased washing temperature. Thus, the use a higher washing temperature leads to a higher signal-to-noise ratio.

EXAMPLE 68

25

Amplification system based on biotin:streptavidin

The experiment was carried out as described in Example 65 using the single-labelled conventional probe of Example 65. In the final visualisation step, a commercial FITC:streptavidin conjugate was compared to plain streptavidin followed by incubation with the dendrimer of Example 16. In the conventional system, one FITC:streptavidin binds to each immobilised biotin. In the dendrimer based system, one streptavidin bind to each biotin, leaving 3 binding sites on streptavidin which can

be exploited for further dendrimer binding. Accordingly, 3 dendrimers of Example 16 with a total of 24 fluorescein molecules binds to each biotin via streptavidin. A signal enhancement of approximately 15 times was observed, 5 consistent with a 1.5 to 1 ratio of FITC to streptavidin in the commercial conjugate.

REFERENCES

1. Wilbur et al., Bioconjugate Chem. 9, 813-825 (1998)
- 5 2. WO 98/32469 (Nycomed Imaging AS)
3. EP 271 180 (The Dow Chemical Company)
4. D. Scott Wilbur et al., Bioconjugate Chem. 9, 813-825
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CLAIMS

1. Dendrimer having the general formula (I)

5 (Entity1)_{x1}-A-(Entity2)_{x2} (I)

wherein A is a dendritic core having at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein A
10 comprises at least one ether group,
each Entity1 and Entity2 are independently a probe, a probe substituted by one or more labelling compounds, a labelling compound, or a probe having reactive groups,
x1 and x2 are independently 0 or an integer of from 1 to
15 1200,
and a protected form thereof.

2. Dendrimer according to claim 1, wherein the dendritic core A extends from a single focal point through multiple generations of successive layers, each layer having one or more branching points.

3. Dendrimer according to claim 1 or 2, wherein the dendritic core A comprises one or more of C₁₋₁₀₀ alkyl groups, C₂₋₁₀₀ alkenyl groups, C₂₋₁₀₀ alkynyl groups, said alkyl, alkenyl, and alkynyl groups optionally containing one or more functional groups and/or one or more heteroatoms, naturally or non-naturally amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences or analogues thereof, RNA sequences or analogues thereof, macromolecules, and solid or semi-solid supports.

4. Dendrimer according to claim 3, wherein the dendritic core A comprises one or more of C_{15-25} alkyl groups, C_{15-25} alkenyl groups, or C_{15-25} alkynyl groups, said alkyl,

alkenyl, and alkynyl groups optionally one or more functional groups and/or one or more heteroatoms.

5. Dendrimer according to claim 3 or 4, wherein the
5 dendritic core A comprises one or more of

$-\text{NH}((\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{N}((\text{CH}_2)_q\text{C}(\text{O})-)_2$,
 $((-\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{N}((\text{CH}_2)_q\text{O}-)_2$,
 $((-\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{C}((\text{CH}_2)_q\text{O}-)_3$, and
 $-\text{NH}((\text{CH}_2)_q\text{O})_q(\text{CH}_2)_q\text{C}((\text{CH}_2)_q\text{C}(\text{O})-)_3$,

10

wherein each q independently is 0 an integer from 1 to 8, preferably from 1 to 3.

6. Dendrimer according to claims 3-5, wherein the
15 dendritic core comprises one or more of

$-\text{NH}(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{C}(\text{O}))_2-$,
 $-\text{NH}(\text{CH}_2)_2\text{NHC}(\text{O})\text{CH}_2(\text{N}(\text{CH}_2\text{CH}_2)\text{N})\text{CH}_2\text{C}(\text{O})-$,
 $(-\text{NH}(\text{CH}_2)_3(\text{O})(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{COOH})\text{CH}_2\text{C}(\text{O}))_q$,

wherein q is as defined above,

20 $-\text{NH}(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{COOH})\text{CH}_2\text{C}(\text{O})-$,

$-\text{NH}(\text{CH}_3)_3(\text{OCH}_2\text{CH}_2)_q\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{COOH})\text{CH}_2\text{C}(\text{O})-$, and

$-\text{NH}(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{NHC}(\text{O})\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2)_q\text{CH}_2\text{C}(\text{O})\text{NH}-$
 $(\text{CH}_2)_3\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_2\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_2\text{COOH})\text{CH}_2\text{C}(\text{O})-$.

25 7. Dendrimer according to claims 1-6, wherein the interdistance between each Entity1 or Entity2 is at least 150 bonds.

8. Dendrimer according to claims 1-7, wherein one of the
30 Entity1's and the Entity2's is a probe and the other is a labelling compound,
or a protected form thereof.

9. Dendrimer according to claims 1-7, wherein both of the
35 Entity1's and Entity2's are labelling compounds or probes substituted by labelling compounds,

or a protected form thereof.

10. Dendrimer according to claim 9, wherein at least two, at least three, or at least four of the labelling 5 compounds are different labelling compounds, or a protected form thereof.

11. Dendrimer according to claim 9, wherein the Entity1's are a labelling compound or a probe substituted by a 10 labelling compound, and the Entity2's are another labelling compound or a probe substituted by said another labelling compound, or a protected form thereof.

15 12. Dendrimer according to claims 1-7, wherein both of the Entity1's and Entity2's are probes, or a protected form thereof.

13. Dendrimer according to claims 1-12, wherein the 20 labelling compound is selected from fluorophores, biotin, dinitro phenyl radical, digoxigenin, radioisotope labels, or enzyme labels, dyes, chemiluminescence labels, hapten, antigen or antibody labels, and spin labels, or a protected form thereof.

25 14. Dendrimer according to claims 1-12, wherein the probe is selected from peptide nucleic acids, RNA sequences or DNA sequences or analogues thereof, antibodies, antigens, proteins, peptides or derivatives thereof, epitopes, and 30 biotin, or a protected form thereof.

15. Dendrimer according to claims 1-14, wherein both x_1 and x_2 are 0.

16. Dendrimer according to claims 1-14, wherein x1 is 0, and x2 is an integer of from 1 to 1200.

17. Dendrimer according to claims 1-14, wherein x2 is 0, 5 and x1 is an integer of from 1 to 1200.

18. Dendrimer according to claims 1-17, wherein the protecting group providing the protected form is selected from Fmoc, Boc, Mtt, Mmt, Dde, All, Odmab, Aloc, Otbu, 10 OMe, OBz, Z, MOM and benzyloxycarbonyl.

19. Dendrimer according to claims 1-18, wherein x1 and x2 independently is 2^m, and wherein m is an integer of from 1 to 10.

15 20. Dendrimer according to claim 19, wherein m is 2, 3, 4 or 5.

21. Dendrimer according to claims 1-20, wherein the 20 dendritic core A comprises one or more moieties of the structure (Ia)

$Z[(Z)_z()_y]_z$ (Ia)

25 wherein each Z is a group which contains at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein the moiety of formula (Ia) comprises at least one ether group, and wherein each y independently is 2 or 3, and z 30 is an integer of from 1 to 10, with the proviso that y^z ≤ 1200, and a protected form thereof.

22. Dendrimer according to claim 21, wherein the 35 dendritic core A comprises at least two moieties of the structure (Ia) connected to a naturally or non-naturally

amino acid, a peptide nucleic acid moiety, an LNA, a peptide, a protein, an antibody, an antigen, an immune complex, a DNA sequence or an analogue thereof, a RNA sequence or an analogue thereof, a macromolecule, and/or 5 a solid or semi-solid support.

23. Dendrimer according to claim 21 or 22, wherein each Z comprises one or more of C_{1-100} alkyl groups, C_{2-100} alkenyl groups, C_{2-100} alkynyl groups, said alkyl, alkenyl, and 10 alkynyl groups optionally containing one or more functional groups and/or one or more heteroatoms, naturally or non-naturally amino acids, peptide nucleic acid moieties, LNAs, peptides, proteins, antibodies, antigens, immune complexes, DNA sequences and analogues 15 thereof, RNA sequences and analogues thereof, and macromolecules.

24. Dendrimer according to claim 23, wherein the dendritic core A comprises one or more of C_{15-25} alkyl groups, C_{15-25} alkenyl groups, of C_{15-25} alkynyl groups, said alkyl, alkenyl, and alkynyl groups optionally one or 20 more functional groups and/or one or more heteroatoms.

25. Dendrimer according to claim 23 or 24, wherein the 25 dendritic core A comprises one or more of
-NH((CH₂)_qO)_q(CH₂)_qN((CH₂)_qC(O)-)₂,
((-CH₂)_qO)_q(CH₂)_qN((CH₂)_qO-)₂,
((-CH₂)_qO)_q(CH₂)_qC((CH₂)_qO-)₃, and
-NH((CH₂)_qO)_q(CH₂)_qC((CH₂)_qC(O)-)₃,
30 wherein each q independently is 0 an integer from 1 to 8, preferably from 1 to 3.

26. Dendrimer according to claims 21-26, wherein the dendritic core A comprises one or more of
35 -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂C(O))₂-,
-NH(CH₂)₂NHC(O)CH₂(N(CH₂CH₂)N)CH₂C(O)-

(-NH(CH₂)₃(O)(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂COOH)CH₂C(O))_q,

wherein q is as defined above,

-NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(COOH)CH₂C(O)-,

-NH(CH₃)₃(OCH₂CH₂)_qO(CH₂)₃N(CH₂COOH)CH₂C(O)-, and

5 -NH(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃NHC(O)CH₂O(CH₂CH₂)_qCH₂C(O)NH-(CH₂)₃O(CH₂)₂O(CH₂)₂O(CH₂)₃N(CH₂COOH)CH₂C(O)-.

27. Dendrimer according to claims 21-26, wherein the interdistance between each Entity1 or Entity2 is at least
10 150 bonds.

28. Dendrimer according to claims 21-27, wherein one of the Entity1's and the Entity2's is a probe and the other is a labelling compound,
15 or a protected form thereof.

29. Dendrimer according to claims 21-27, wherein both of the Entity1's and Entity2's are labelling compounds or probes substituted by labelling compounds,
20 or a protected form thereof.

30. Dendrimer according to claim 29, wherein at least two, at least three, or at least four of the labelling compounds are different labelling compounds,
25 or a protected form thereof.

31. Dendrimer according to claim 29, wherein the Entity1's are a labelling compound or a probe substituted by a labelling compound, and the Entity2's are another
30 labelling compound or a probe substituted by said another labelling compound,
or a protected form thereof.

32. Dendrimer according to claims 21-27, wherein both of the Entity1's and the Entity2's are probes,
35 or a protected form thereof.

33. Dendrimer according to claims 21-32, wherein the labelling compound is selected from fluorophores, biotin, dinitro phenyl radical, digoxigenin, radioisotope labels, 5 or enzyme labels, dyes, chemiluminiscence labels, hapten, antigen or antibody labels, and spin labels, or a protected form thereof.

34. Dendrimer according to claims 21-32, wherein the 10 probe is selected from peptide nucleic acids, RNA sequences or DNA sequences or analogues thereof, antibodies, antigens, proteins, peptides or derivatives thereof, epitopes, and biotin, or a protected form thereof.

15

35. Dendrimer according to claims 21-34, wherein both x_1 and x_2 are 0.

36. Dendrimer according to claims 21-34, wherein x_1 is 0, 20 and x_2 is an integer of from 1 to 1200.

37. Dendrimer according to claims 21-34, wherein x_2 is 0, and x_1 is an integer of from 1 to 1200.

25 38. Dendrimer according to claims 21-37, wherein x_1 and x_2 independently is 2^m , and wherein m is an integer of from 1 to 10.

30 39. Dendrimer according to claim 38, wherein m is 2, 3, 4 or 5.

40. Dendrimer according to claims 1-39, wherein at least one of the Entity1's and the Entity2's is connected to a naturally or non-naturally amino acid terminally or 35 internally, a peptide nucleic acid terminally or internally, a peptide terminally or internally, an LNA

terminally or internally, a protein terminally or internally, an antibody, an antigen, an immune complex, a RNA sequence or an analogues thereof terminally or internally, a DNA sequence or an analogue thereof 5 terminally or internally, a macromolecule terminally or internally, or a solid or semi-solid support.

41. Protected dendrimer according to claims 1-40.

10 42. Dendrimer complex comprising at least one dendrimer according to claims 1-41 connected to a naturally or non-naturally amino acid terminally or internally, a peptide nucleic acid terminally or internally, an LNA terminally or internally, a peptide terminally or internally, a protein terminally or internally, an antibody, an antigen, an immune complex, a RNA sequence or an analogue thereof terminally or internally, a DNA sequence or an analogue terminally or internally, a macromolecule terminally or internally, or a solid or semi-solid 15 support,
20 or a protected form thereof.

43. Dendrimer complex according to claim 42, wherein the dendrimer is connected via at least one of the Entity1's and/or the Entity2's.
25

44. Dendrimer complex according to claim 42, wherein the dendrimer is connected via a group or groups of the dendritic core A.
30

45. Dendrimer complex according to claims 42-44, wherein the dendrimer complex comprises 1-50 dendrimers according to claims 1-41.

46. Dendrimer complex according to claim 45, wherein the dendrimer complex comprises 1-35, 1-30, 1-25, 1-15, 1-10, 1-8, 1-5, or 1-3 dendrimers according to claims 1-41.

5 47. Dendrimer complex according to claims 42-46, wherein the dendrimer is internally connected to a naturally or non-naturally occurring amino acid, a peptide nucleic acid, an LNA, a protein, a RNA sequence or a DNA sequence, a macromolecule, or a solid or a semi-solid .
10 support.

48. Protected dendrimer complex according to claims 42-47.

15 49. Use of a dendrimer according to claims 1-41 or a dendrimer complex according to claims 42-48 for detecting the presence of nucleic acid sequences, antibodies, antigens, immune complexes, proteins, or peptides in a sample.

20 50. A detection system comprising at least one dendrimer according to claims 1-41 and/or at least one dendrimer complex according to claims 42-48.

25 51. A signal amplification system comprising at least one dendrimer according to claims 1-41 and/or at least one dendrimer complex according to claims 42-48.

30 52. Use of a dendrimer according to claims 1-41 or a dendrimer complex according to claims 42-48 as a detection system.

35 53. Use of a dendrimer according to claims 1-41 or a dendrimer complex according to claims 42-48 as a signal amplification system.

54. Use of the detection system according to claim 50 or the amplification system according to claim 51 for detecting the presence of nucleic acid sequences, antibodies, antigens, immune complexes, proteins, or 5 peptides in a sample.

55. Use according to claim 54, wherein the sample is a blood sample, a bone marrow sample, a chromosome spread, a tissue sample, a tissue section, a cell smear, a 10 biopsy, an organ, a swap, a suspension of cells or parts thereof, or a whole cell or parts thereof.

56. Use of a dendrimer according to claims 1-41 or a dendrimer complex according to claims 42-48 in an in 15 vitro diagnostic method or an in vivo diagnostic method.

57. Use of a dendrimer according to claims 1-41 or a dendrimer according to claims 42-48 for labelling a compound.

20 58. Use according to claim 57, wherein the compound to be labelled is selected from peptide nucleic acids, LNAs, RNA sequences, DNA sequences or analogues thereof, antibodies, antigens, proteins, peptides or derivatives 25 thereof, epitopes, streptavidin, and biotin.

59. Use according to claims 57 or 58, wherein the dendrimer or dendrimer complex comprises one or more labelling compounds selected from fluorophores, biotin, 30 dinitro phenyl radicals, digoxigenin, radioisotope labels, enzyme labels, dyes, chemiluminescence labels, haptens, antigen or antibody labels, and spin labels.

60. Labelling kit for labelling a compound comprising one 35 or more dendrimers according to claims 1-41 and/or dendrimer complexes according to claims 42-48.

61. Labelling kit according to claim 60, further comprising a labelling compound.

5 62. Labelling kit according to claim 60, wherein the dendrimer or dendrimer complex comprises a labelling compound.

10 63. Labelling kit according to claim 60 or 61, wherein the labelling compound is selected from fluorophores, biotin, dinitro phenyl radicals, digoxigenin, radioisotope labels, enzyme labels, dyes, chemiluminiscence labels, haptens, antigen or antibody labels, and spin labels.

15 64. Detection kit for detecting a compound comprising one or more dendrimers according to claims 1-42 and/or a dendrimer complex according to claims 42-48, wherein the dendrimer and/or the dendrimer complex comprise(s) one or 20 more labelling compounds.

25 65. Detection kit according to claim 64, wherein the labelling compound is selected from fluorophores, biotin, dinitro phenyl radicals, digoxigenin, radioisotope labels, enzyme labels, dyes, chemiluminiscence labels, haptens, antigen or antibody labels, and spin labels.

30 66. A process for preparing a dendrimer having the general formula (I)

(Entity1)_{x1}-A-(Entity2)_{x2} (I)

35 wherein A is a dendritic core having at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein A comprises at least one ether group,

each Entity1 and Entity2 are independently probes which optionally are substituted by one or more labelling compounds, a labelling compound, or a probe having reactive groups,

5 x1 and x2 are independently 0 or an integer of from 1 to 1200,

or a protected form thereof
comprising

10 preparing a dendritic core A by chemical/peptide synthesis, depending on the components of the dendritic core A, which dendritic core A has a number of protecting groups, which can be removed by deprotection, if desired, removing the protecting groups by use of a
15 suitable deprotecting agent,
if desired, coupling the Entity1's and/or the Entity2's to the dendritic core A, thereby obtaining the dendrimer or the protected form thereof.

20 67. A process for preparing a dendrimer having the
general formula (I)

(Entity1)_{x1}-A-(Entity2)_{x2} (I)

25 wherein A is a dendritic core having at least one N-atomic branching point, said branching point not being part of a naturally occurring amino acid, and wherein A comprises at least one ether group,

each Entity1 and Entity2 are independently probes which
optionally are substituted by one or more labelling
compounds, a labelling compound, or a probe having
reactive groups,

x1 and x2 are independently 0 or an integer of from 1 to 1200,

35 or a protected form thereof,
comprising

preparing building blocks for the dendritic core A by chemical/peptide synthesis, depending on the components of the dendritic core A,

5 assembling the dendritic core A, which dendritic core A has a number of protecting groups, which can be removed by deprotection,
if desired, removing the protecting groups by use of a suitable deprotecting agent,

10 if desired, coupling the Entity1's and/or the Entity2's to the dendritic core A, thereby obtaining the dendrimer or the protected form thereof.

68. Dendrimer obtainable by the process according to
15 claim 66 or 67.

Examples of protecting groups.

Boc-

Fmoc-

Mtt-

Benzylloxycarbonyl-

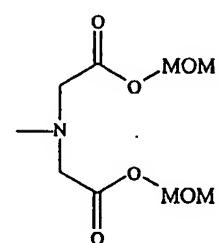
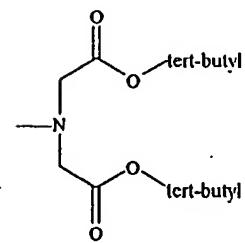
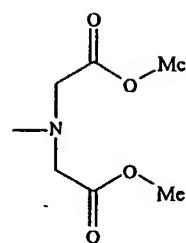
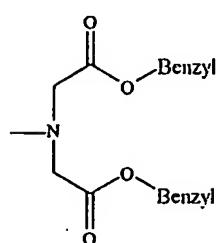


Figure 1

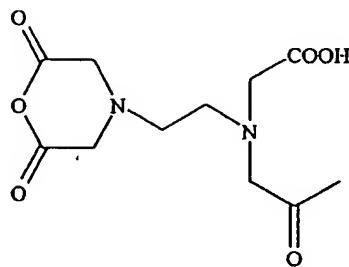
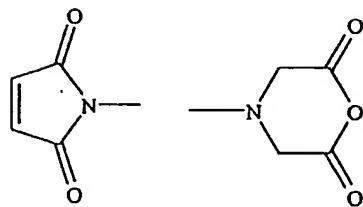
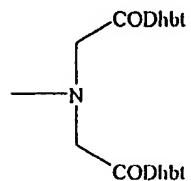
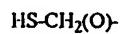
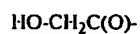
Examples of activated groups (after deprotection)

Figure 2

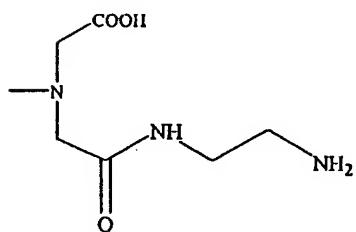
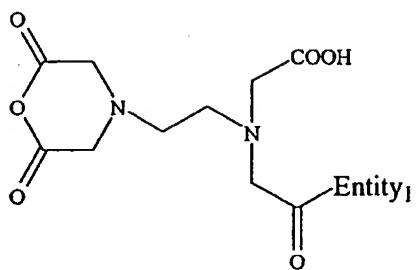
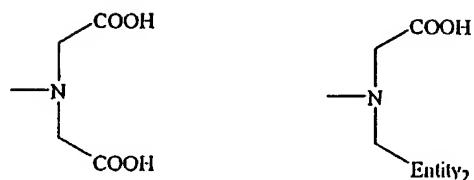
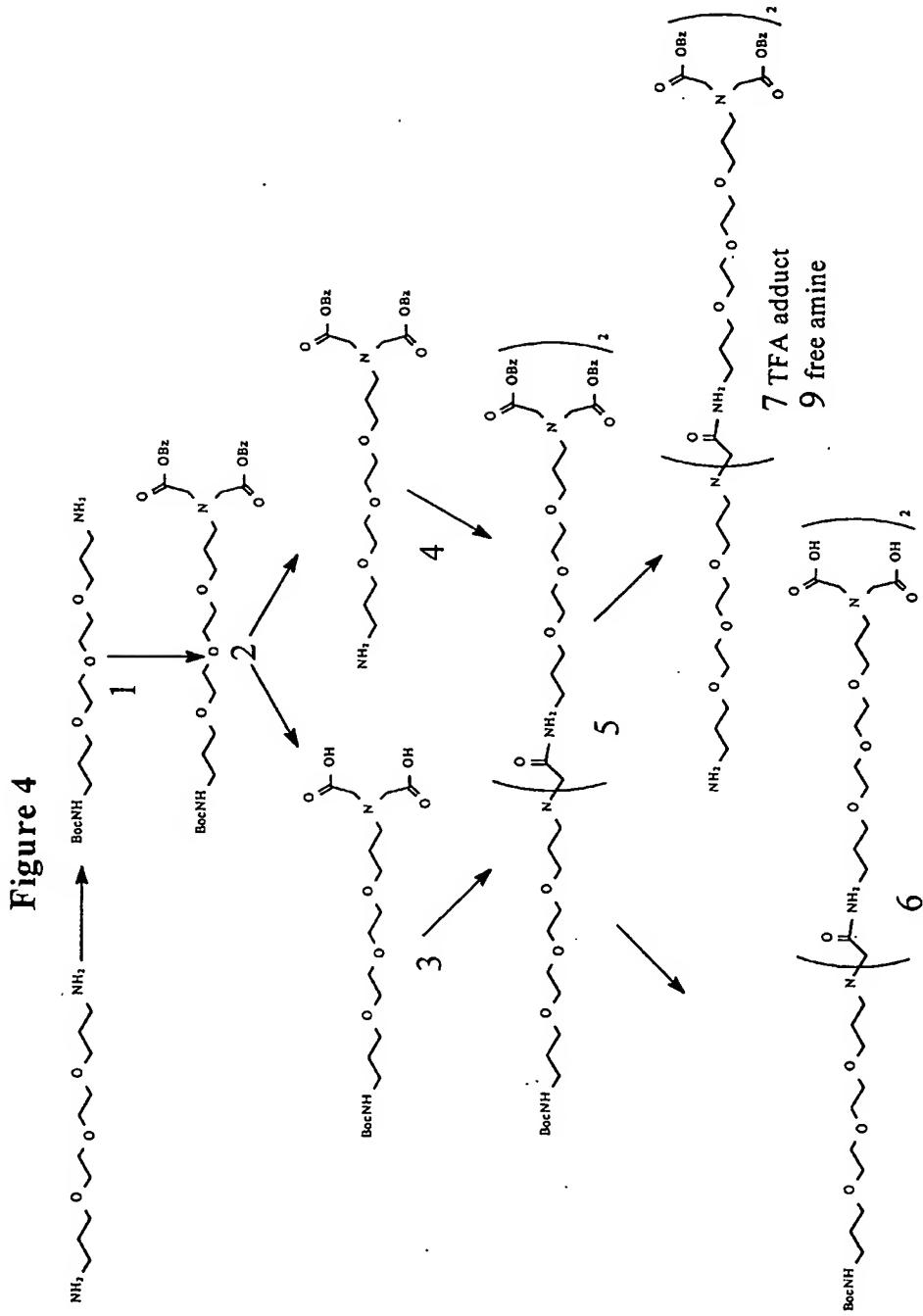
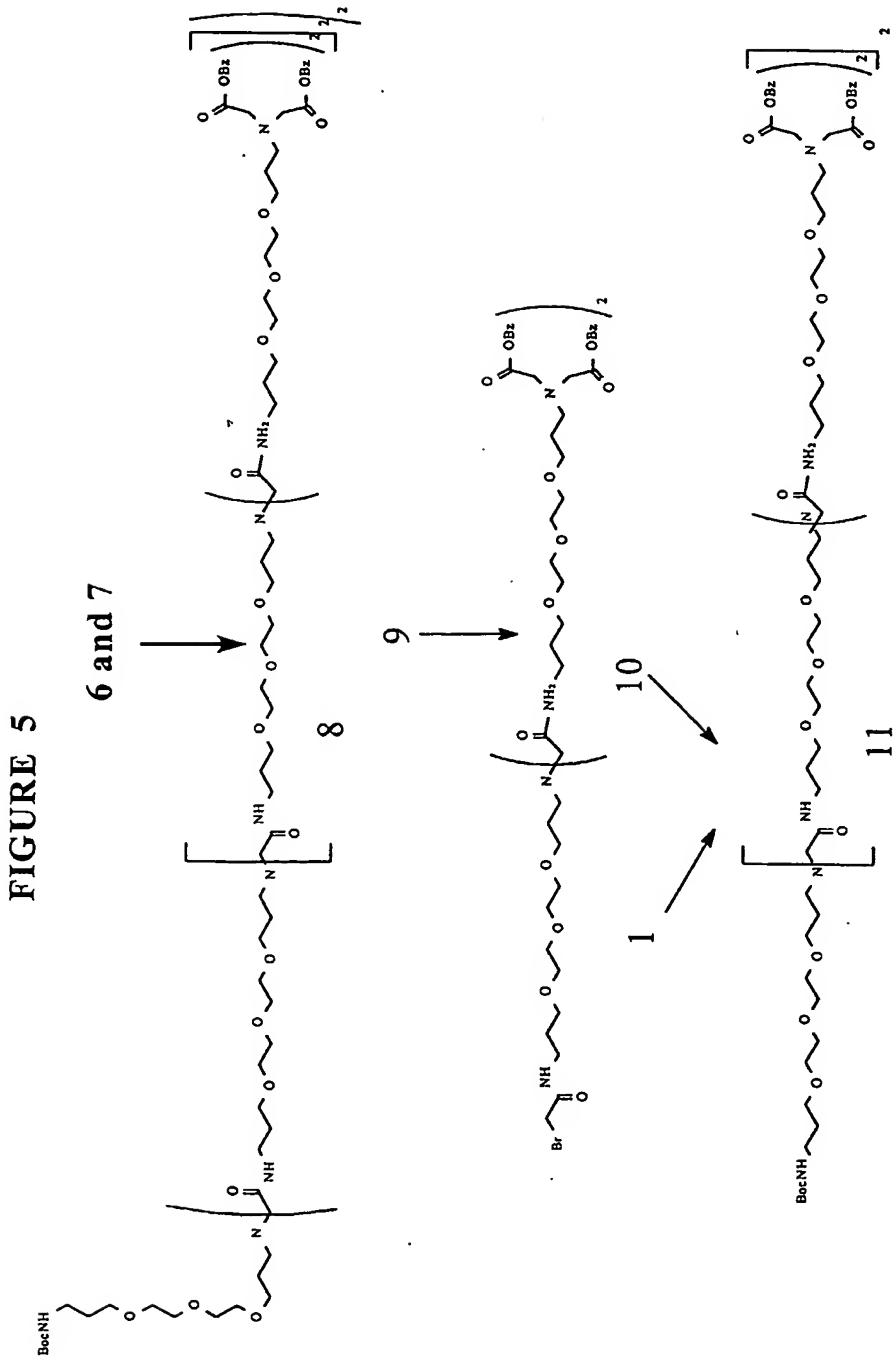
Examples of free groups -NH_2 -COOH 

Figure 3

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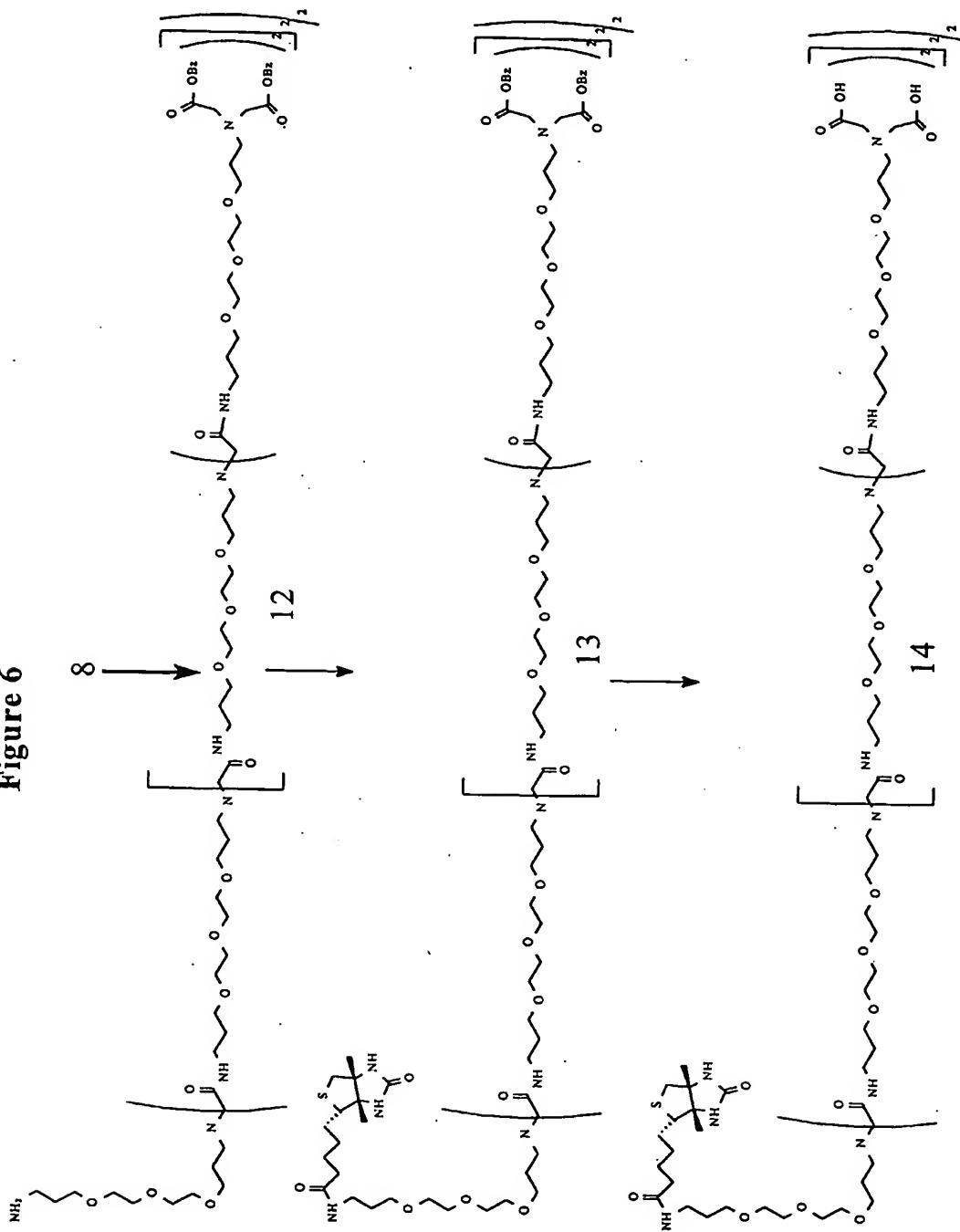


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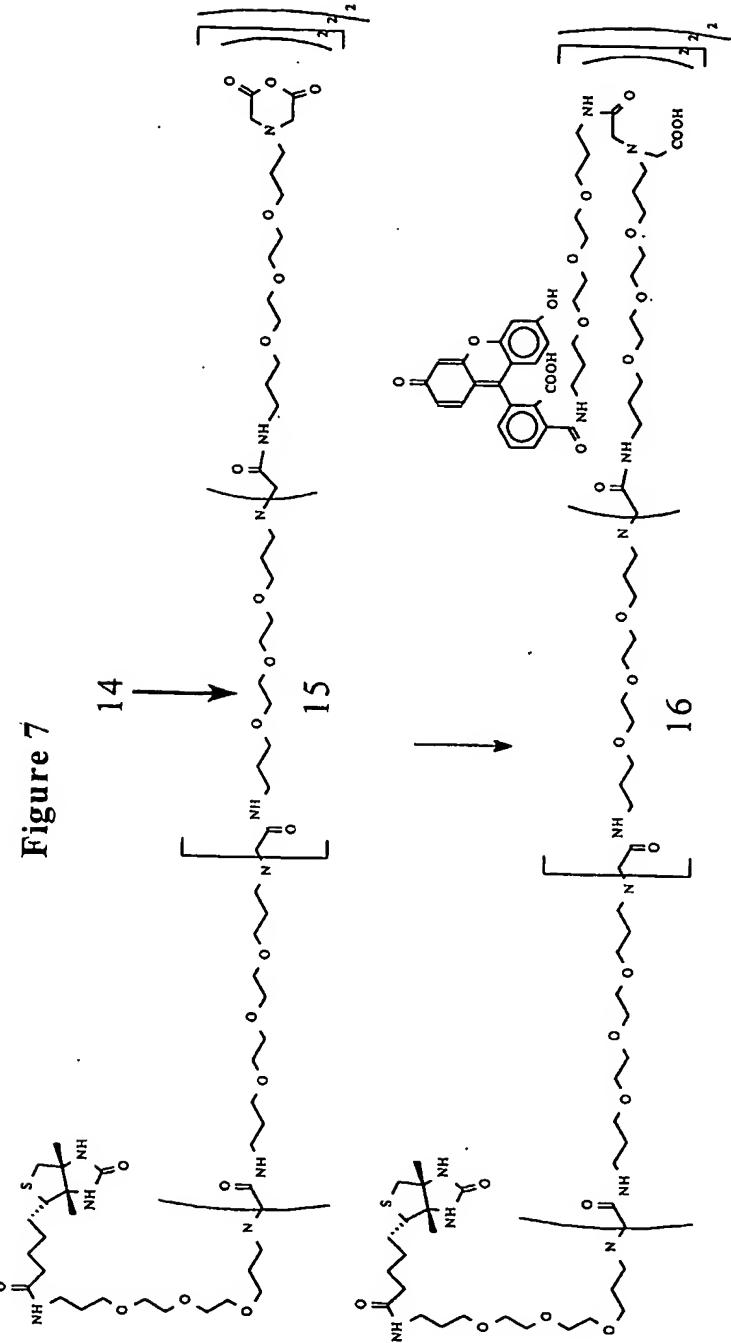


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Figure 6



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Figure 8

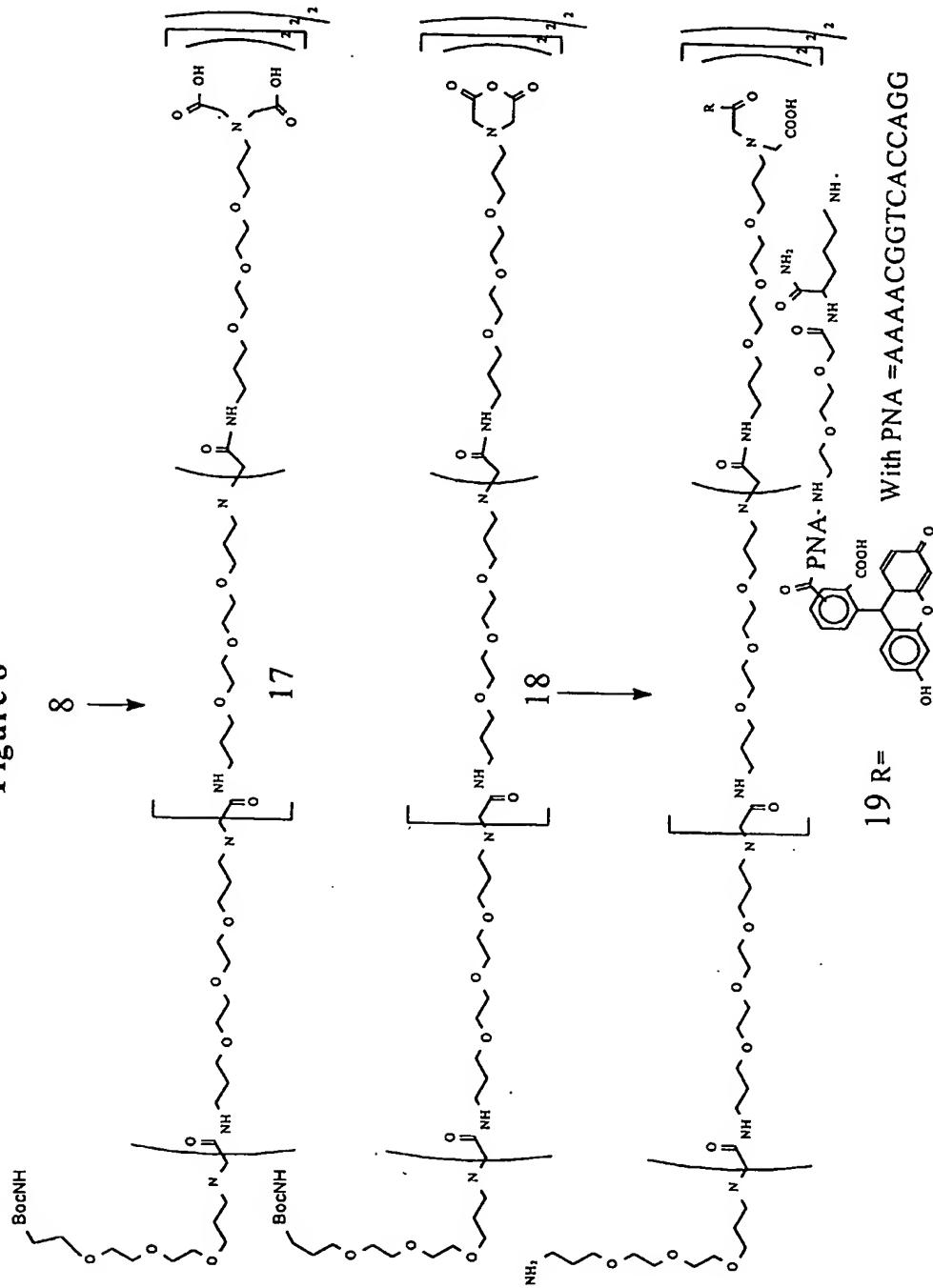
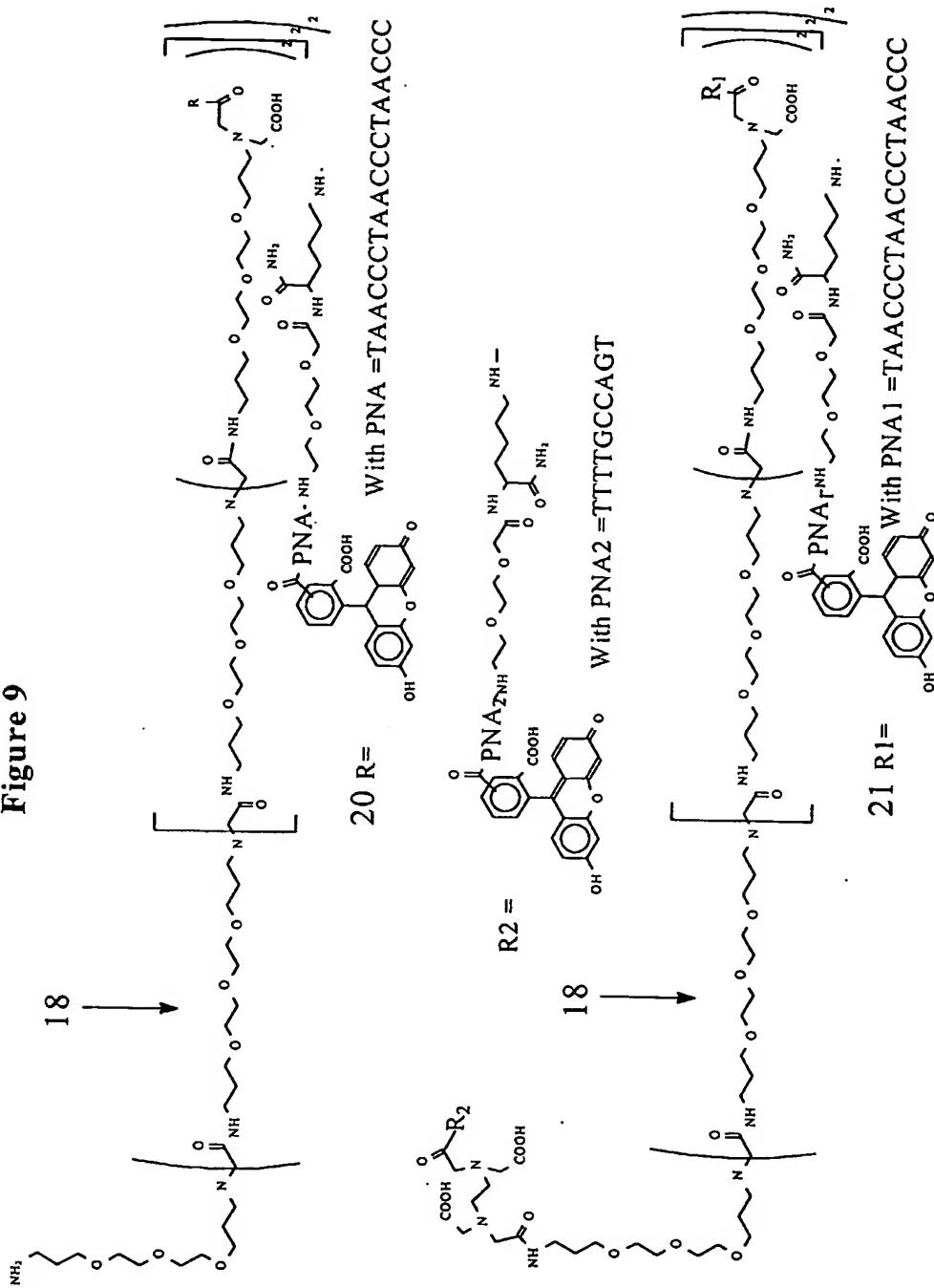
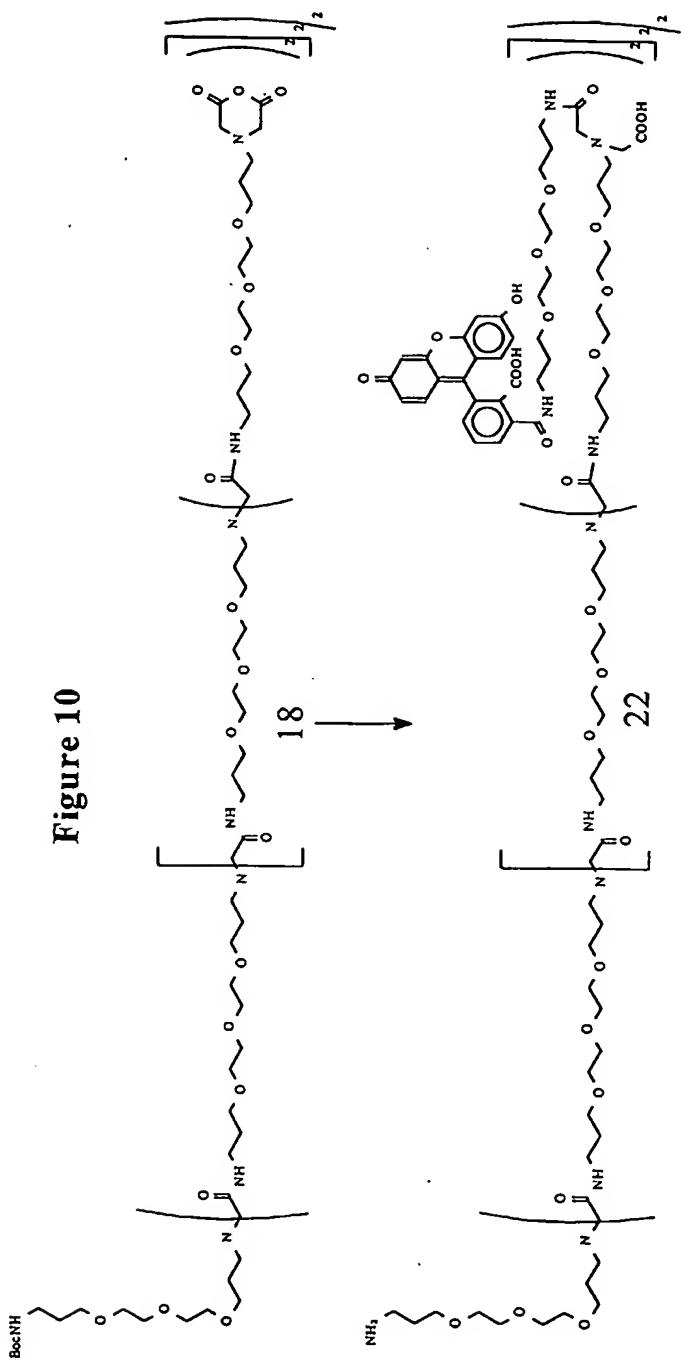


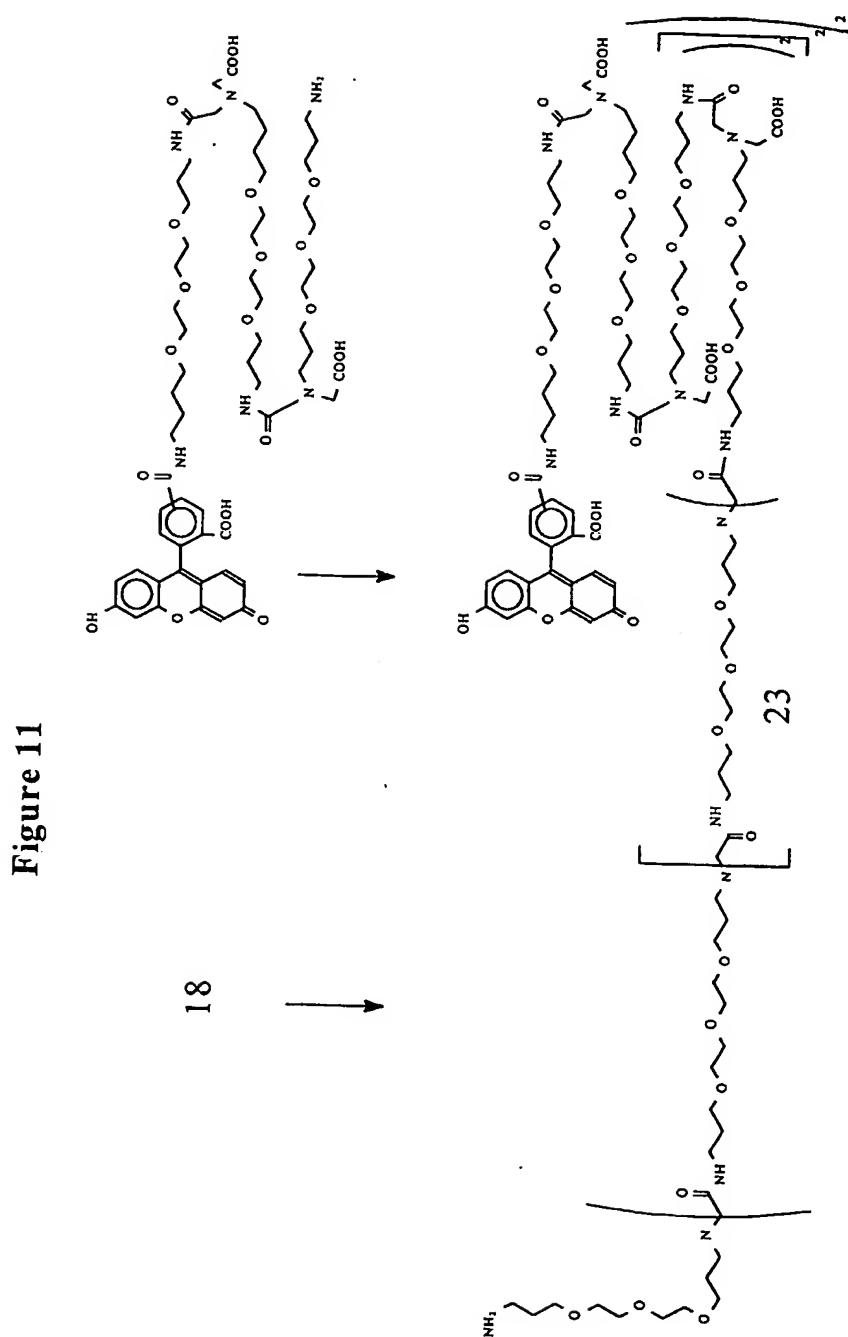
Figure 9



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Figure 12

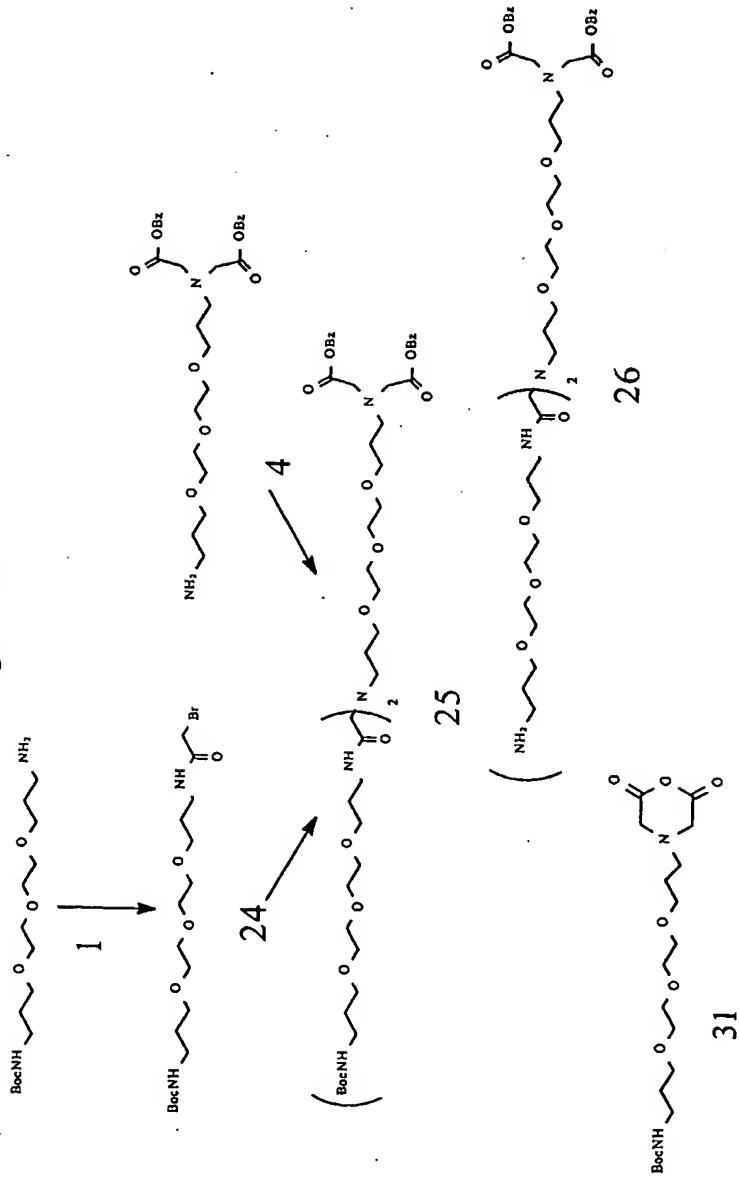
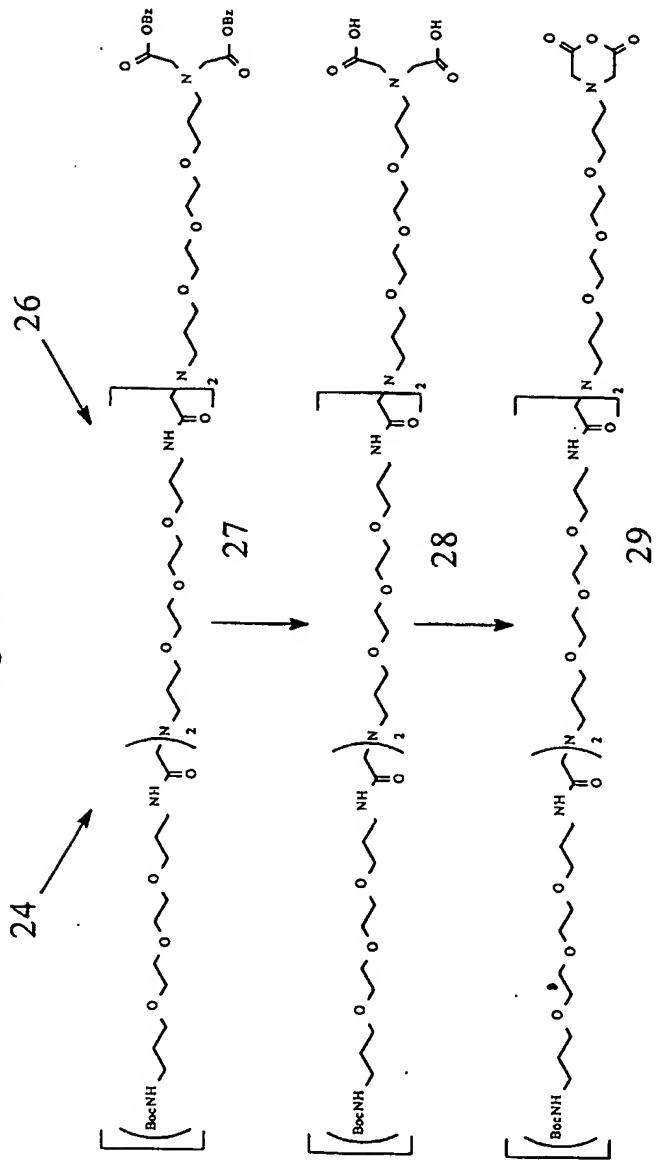
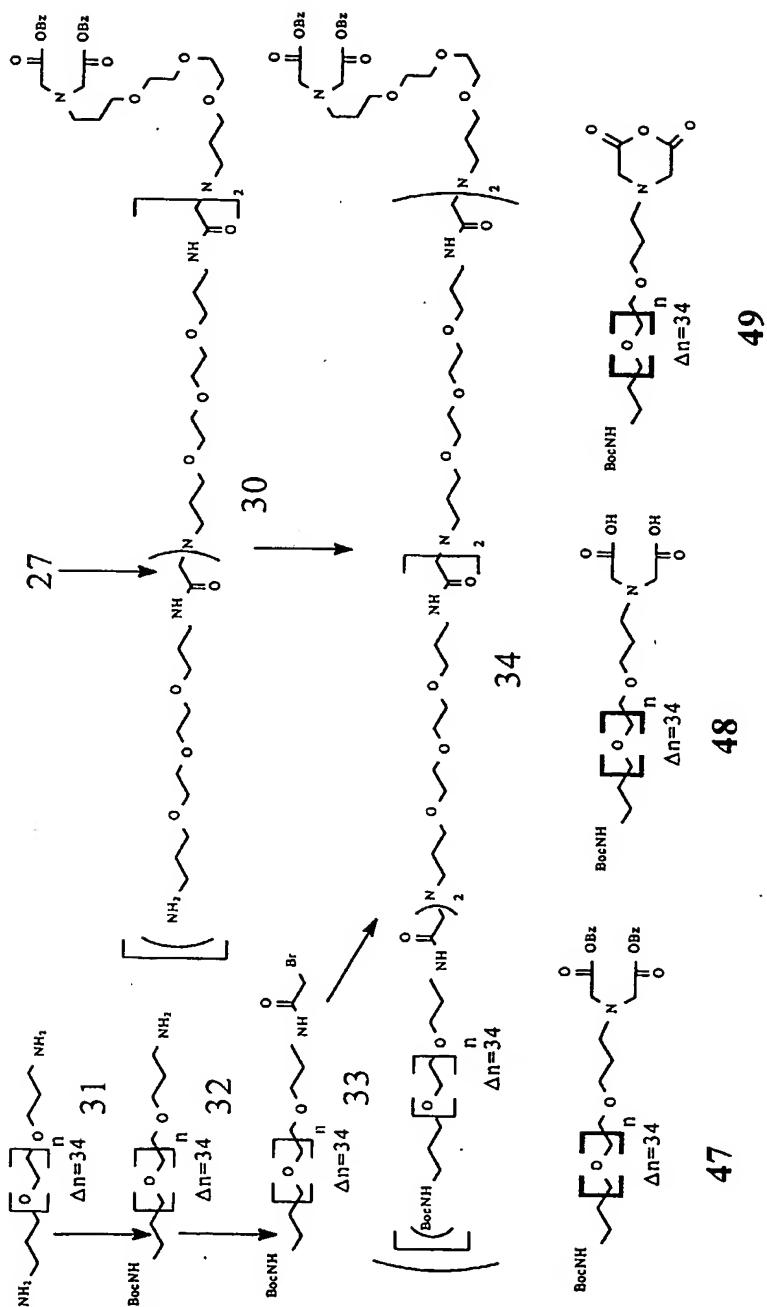


Figure 13



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Figure 14



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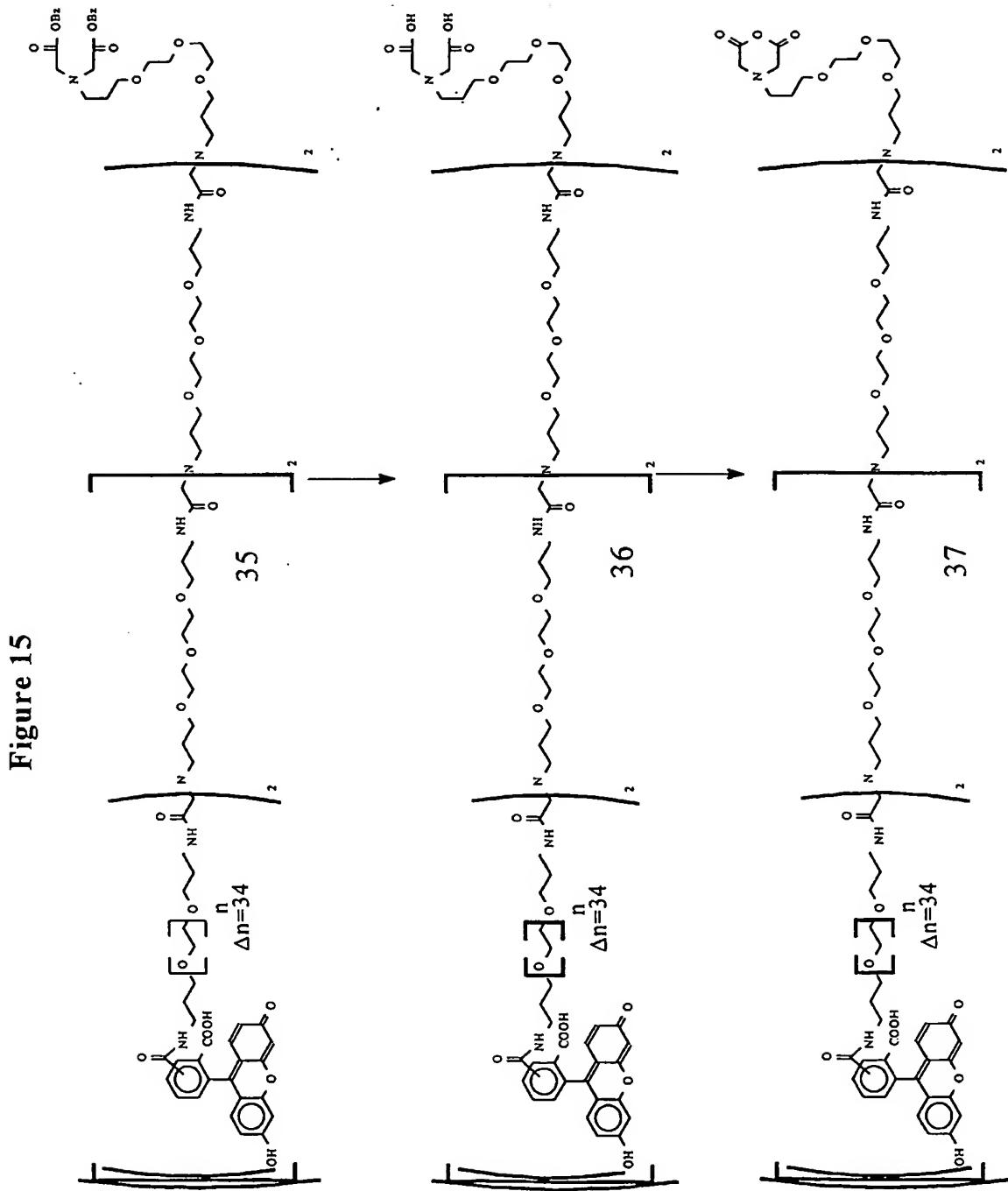


Figure 16

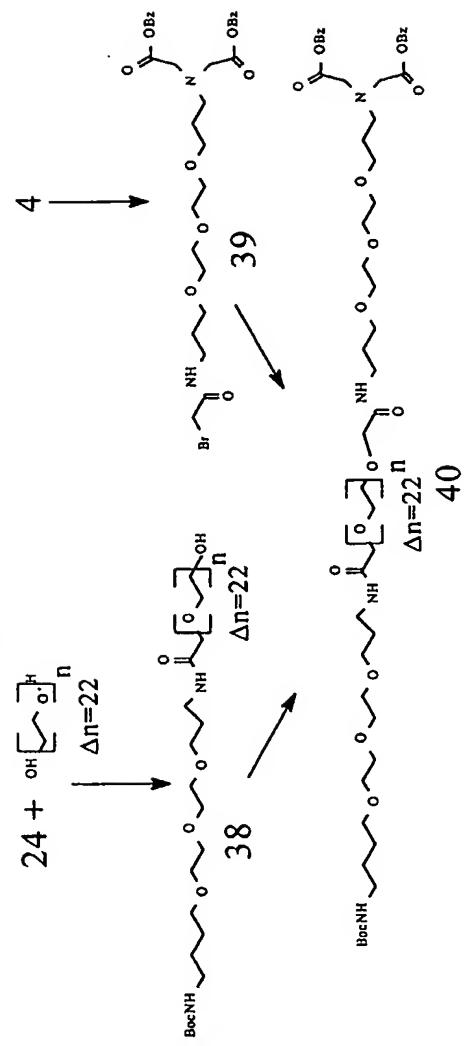
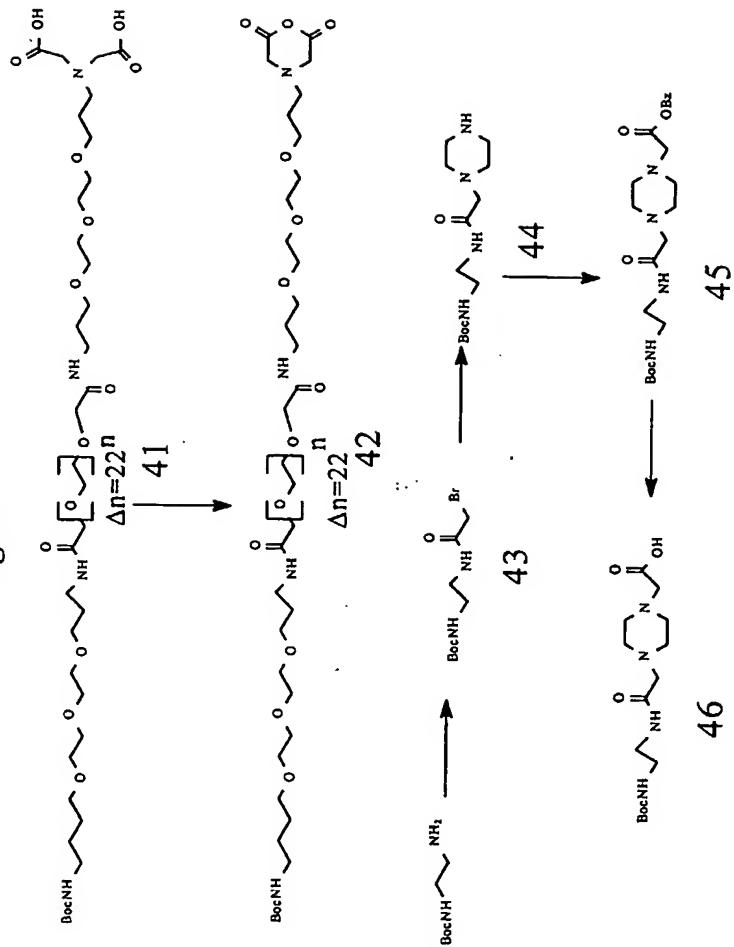


Figure 17



INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 00/00351

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/58 C12Q1/68 C08G83/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 00 55111 A (SCOTT DAVID ;FINN MALCOLM (GB); FLEET GEORGE (GB); ISIS INNOVATION) 21 September 2000 (2000-09-21) page 39 -page 41 ---	1-68
A	WO 98 32469 A (GOLDING LOUISE ;NYCOMED IMAGING AS (NO); WOLFE HENRY (US); KELLAR) 30 July 1998 (1998-07-30) cited in the application figures 1A,1B ---	1-68 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the International search

15 November 2000

Date of mailing of the International search report

24/11/2000

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Hart-Davis, J

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/DK 00/00351

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	WO 97 07398 A (DADE INT INC) 27 February 1997 (1997-02-27) cited in the application abstract ---	1-68
A	WILBUR, D. S.; HAMLIN, D. K.; PAHHARE, P. M.; WEERAWARNA, S. A.: "Investigation of Biotin Dimers and Trimers for increasing the Quantity of Radioactivity on Cancer Cells in Tumor Pretargeting. In Vitro Demonstration of streptavidin Cross-linking" JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS, vol. 40, 1997, pages 335-337, XP000856594 table 1 ---	1,5,6, 25,26
A	P SINGH, F MOLL, S H LIN, C FERZLI, K S YU, R K KOSKI, R G SAUL, P CRONIN: "Starburst Dendrimers: Enhanced Performance and Flexibility" CLINICAL CHEMISTRY, vol. 40, no. 9, 1994, pages 1845-1849, XP002124248 figure 1 ---	1-68
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INTERNATIONAL SEARCH REPORT

Inten.ional Application No

PCT/DK 00/00351

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Intellinal Application No

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